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Vliv neurosteroidů na aktivitu neuronální sítě *in vitro*.

Neurosteroid effects on neuronal network activity *in vitro*.

Diplomová práce

Vedoucí diplomové práce: Tereza Smejkalová, Ph.D.

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Poděkování

Tímto bych chtěla poděkovat své školitelce Tereze Smejkalové, Ph.D. za trpělivou pomoc, vstřícný přístup a odborné vedení diplomového projektu. Dále bych chtěla poděkovat své rodině a přátelům za jejich oporu během celého vysokoškolského studia i v průběhu vypracovávání této závěrečné práce. V neposlední řadě bych ráda poděkovala panu RNDr. Janu Krůškovi, Ph.D. za vyhotovení aplikačního systému, odbornou pomoc a cenné rady k experimentální práci. V neposlední řadě pak děkuji všem dalším kolegům z oddělení Buněčné neurofyziologie za vytvoření přátelského pracovního kolektivu.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 11. 8. 2021

Podpis

Abstrakt

GABA receptory typu A ($GABA_AR$) jsou ligandem řízené iontové kanály propustné pro chloridové anionty. V savčím mozku zprostředkovávají většinu inhibiční neurotransmise a poruchy v GABAergním systému mají za následek řadu neurologických poruch, jakými jsou epilepsie, úzkosti a deprese. Neurosteroidy jsou metabolity cholesterolu, které účinkují na řadu membránových receptorů a mají přímý vliv na neuronální excitabilitu. Neurosteroidy allo-pregnanolon (allo-PA) a pregnanolon (PA) jsou silné pozitivní modulátory $GABA_AR$. Cílem této práce bylo zavést aplikační systém a miktrospektrofluorimetrickou metodu měření změn v koncentraci somatického vápníku (tzv. calcium imaging) s využitím senzoru GCaMP a pomocí ní otestovat vliv PA na aktivitu primárních hipokampálních kultur. Zavedli jsme aplikační systém a otestovali vápníkový senzor GCaMP *in vitro*. Aplikace PA inhibovala spontánní vápníkové vrcholy, což souhlasí s jeho známým účinkem na $GABA_AR$. Zjistili jsme, že při opakované aplikaci neurosteroidu došlo ke změně jeho inhibičního účinku na aktivitu neuronální sítě. Naše výsledky naznačují, že při dlouhodobém či opakovaném vystavení neuronů PA může docházet k určitým kompenzačním mechanismům na úrovni $GABA_AR$.

Klíčová slova: $GABA_AR$; neurosteroid; pregnanolon; allopregnanolon; vápník; GCaMP; inhibice

Abstract

GABA receptors type A ($GABA_AR$) are ligand-gated ion channels permeable for chloride anions. In the mammalian brain they mediate most of the inhibitory transmission. Moreover, the dysfunctions of the GABA-mediated system result in many neurological disorders, including epilepsy, anxiety and depression. Neurosteroids are cholesterol metabolites interacting with a variety of membrane receptors and have a direct effect on neuronal excitability. The neurosteroids allo-pregnanolone (allo-PA) and pregnanolone (PA) are potent positive modulators of the $GABA_AR$. The goal of this work is to establish a newly constructed application system and a calcium imaging method using the GCaMP sensor to examine the effects of PA on the activity of primary hippocampal cultures. In this work we validate the application system and test the GCaMP calcium sensor *in vitro*. Application of PA inhibited the spontaneous calcium peaks, which agrees with its known actions on the $GABA_AR$. We discovered that the neurosteroid inhibitory effect on the neuronal network activity changes after repeated applications. The results suggest that there might be some compensatory actions on the $GABA_AR$ level during prolonged or repeated exposition to PA.

Key words: $GABA_AR$; neurosteroid; pregnanolone; allopregnanolone, calcium; GCaMP; inhibition

Abbreviations

AAV9	adeno-associated virus 9
allo-PA	allopregnanolone
AP	action potential
AraC	β-D-arabinofuranosid
asparagine 407	Asn 407
AUC	area under curve
Ca^{2+}	calcium ion
CaM	calmodulin
CBP	calmodulin-binding peptide
Cl^-	chloride ion
$^{36}Cl^-$	radioactively labeled Cl^-
CTR	control
DGGC	dentate gyrus granule cell
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ECD	extracellular domain
FBS	fetal bovine serum
ECS	extracellular solution
FDA	Food and Drug Administration
ΔF/F	fluorescence intensity over basal fluorescence
GABA	gamma-aminobutyric acid
$GABA_AR$	GABA receptor type A

$GABA_BR$	GABA receptor type B
GFP	green fluorescent protein
glutamine 241	Gln 241
HAM-D	Hamilton Rating Scale for Depression
HEK 293	human embryonic kidney 293 cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICD	intracellular domain
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potentials
jGCaMP7	GCaMP7 developed at the Janelia Research Campus
MDD	major depressive disorder
mIPSC	miniature IPSC
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
P0 – P2	postnatal day 0 – 2
PA	pregnanolone
pen/strep	penicillin/streptomycin
PLGIC	pentameric ligand-gated ion channels
PPD	postpartum depression
PTSD	posttraumatic disorder
ROI	region of interest
Ro 15-1788	flumazenil
sIPSC	spontaneous IPSC
sCMOS	scientific Complementary metal–oxide–semiconductor

SR95531	gabazine
[^{35}S]TBPS	*t*-butylbicyclophosphorothionate
THDOC	tetrahydrodeoxycorticosterone
TM (or M)	transmembrane helix
TTX	tetrodotoxin
threonine 236	Thr 236
tyrosine 284	Tyr 284
tyrosine 410	Tyr 410

Contents

1. Introduction

Neurosteroids, or neuroactive steroids, are cholesterol metabolites synthetized directly in the central nervous system (Corpechot et al., 1981; Schverer et al., 2018). In contrast to classic peripheral steroid hormones, neurosteroids mainly interact with membrane receptors and can rapidly affect neuronal excitability (Lloyd-Evans & Waller-Evans, 2020).

Among the most notable effects of neurosteroids are positive and negative modulations of ionotropic neurotransmitter receptors, such as the gamma-aminobutyric acid receptors type A ($GABA_AR$) (Lambert et al., 2009). $GABA_AR$ account for most of the inhibitory neurotransmission in the mammalian brain and the dysfunction of the GABA-mediated system leads to several neurological and psychiatric disorders, including epilepsy, anxiety and major depressive disorder (Zorumski et al., 2019).

The derivatives of progesterone, allo-pregnanolone (allo-PA) and pregnanolone (PA), are endogenous positive modulators of $GABA_AR$ (Harrison et al., 1987; Majewska et al., 1986). The imbalance of these endogenous neurosteroids has been connected to stress and symptoms of postpartum depression (PPD) (Meltzer-Brody & Kanes, 2020). The recent approval of allopregnanolone as a therapy for PPD by the American Food and Drug Administration only highlights the clinical importance of understanding the actions of the $GABA_AR$-modulating neurosteroids (Meltzer-Brody et al., 2018; Meltzer-Brody & Kanes, 2020). It is especially essential to examine how these compounds affect the overall activity of neuronal networks at longer timescales.

Examining the effects of neurosteroids on neuronal network activity of primary neuronal cultures represents a stepping stone between electrophysiology recordings and *in vivo* behavioral experiments. Calcium imaging is a relatively noninvasive method that enables us to study the spontaneous activity of neuronal networks by measuring the changes of intracellular Ca^{2+} concentration that are connected to electrical activity. Genetically encoded calcium indicators (Pérez Koldenkova & Nagai, 2013), such as the GCaMP calcium sensor, can detect even very fast and small changes in the Ca^{2+} concentration and their use can be adapted to *in vitro*, as well as *in vivo* model systems, which makes them an extremely useful experimental tool (Dana et al., 2019).

2. Neurosteroids and their function in the brain

Neurosteroids can be synthetized in neurons and glial cells either *de novo* from cholesterol, or from other steroidal precursors transported from peripheral tissues (Lloyd-Evans & Waller-Evans, 2020). Depending on the character of their action on the respective membrane receptors, they can be categorized as positive or negative modulators. The most common and extensively studied neurosteroid targets include N-methyl-D-aspartate receptors (NMDAR) and $GABA_AR$, but acetylcholine nicotinic receptors, voltage-gated calcium channels and other types of membrane receptors are also effected by neurosteroids (Dubrovsky, 2005). It is worth noting that neurosteroids often have multiple targets and actions which result in opposing outcomes, and it is therefore important to take into account their overall effect on neuronal function.

2.1. Allo-PA and PA: structure and biosynthesis

In brief, the biosynthesis of allo-PA and PA begins with cholesterol which gets transported to the mitochondria and is consequently converted by the cytochrome P450, residing in the inner mitochondrial membrane, to pregnenolone. Pregnenolone is then transformed to progesterone in the cell cytoplasm by the 3β-hydroxysteroid dehydrogenase enzyme. In some cases, the synthesis of the neurosteroids can begin with progesterone or other steroid precursors transported to the brain by the plasma from the periphery and crossing the blood-brain barrier (Lloyd-Evans & Waller-Evans, 2020). Then by a series of enzymatic steps 3α,5α-tetrahydroprogesterone and 3α,5β-tetrahydroprogesterone (allo-PA and PA) get synthetized (Lloyd-Evans & Waller-Evans, 2020).

Allo-PA and PA share a very similar molecular structure, with the only difference being the orientation of one hydrogen atom on the fifth carbon of the steroid skeleton. The general structure of $GABA_AR$-modulating pregnane neurosteroids is described in Figure 1. Structural similarity of the two compounds corresponds to the similarity of their effects, with only a slightly larger potency of allo-PA (Harrison et al., 1987), and we are therefore going to describe them together in this thesis, unless stated otherwise.

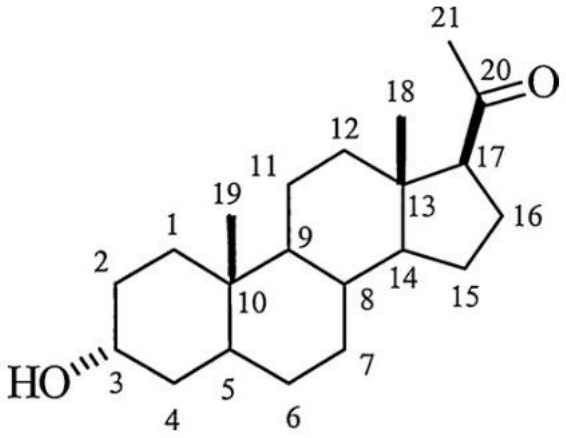

Figure 1: General chemical structure of 3α-hydroxy neurosteroids. The common feature of the the $GABA_AR$-potentiating neurosteroids is a hydroxyl group at the third carbon (C3) in the α position, which means it is oriented under the steroid plane. Allo-PA has a hydrogen at the C5 oriented in the α position which makes it a 3α,5α isomer, whereas PA has the C5 hydrogen in the β position oriented above the steroid plane, making it a 3α,5β isomer. Adapted from (Zorumski et al., 2013).

3. $GABA_A$ receptors

GABAR represent a major class of inhibitory neurotransmitter receptors in the CNS. They can be divided into two main groups, based on their mechanism of action: ionotropic, chloride (Cl^-) conducting GABA receptors type A ($GABA_AR$), and metabotropic, G protein-coupled GABA receptors type B ($GABA_BR$) (Chua & Chebib, 2017). GABAR are responsible for most inhibitory neurotransmission in the mammalian brain, which is necessary for both short and long-term regulation of neuronal excitability and coordination of brain function (Castellano et al., 2021; Wang, 2011).

$GABA_AR$ act as an important target for neurosteroids and more commonly used or potential therapeutic agents (Wang, 2011). Moreover, research shows that deficits in $GABA_AR$ function cause a great number of neuropathological conditions, ranging from anxiety and depression to epilepsy (Castellano et al., 2021), and it is thus of great interest to understand their biology, as well as the actions of $GABA_AR$ modulating compounds.

$GABA_AR$, along with nicotinic acetylcholine receptors, glycine receptors, and serotonin type 3 receptors, belong to a super-family of so-called Cys-loop receptors, which are pentameric ligand-gated ion channels (PLGIC) (Chua & Chebib, 2017).

The members of the Cys-loop family share a few common features, such as the number and architecture of receptor subunits (Sigel & Steinmann, 2012).

3.1. $GABA_AR$ subunits

To this day, 19 isoforms of $GABA_AR$ subunits have been identified, as reviewed in (Wang, 2011): α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ, and ρ1–3. Each of the receptor subunits contains an intracellular domain, four hydrophobic transmembrane helices (TM) and an extracellular domain (ECD), which includes the so-called Cys loop and both the N- and C-terminals.

The functional properties of $GABA_AR$ are dependent on subunit composition, as well as their arrangement (Sigel & Steinmann, 2012). In recent years, electron microscopy and crystal structures of human $GABA_ARs$ composed of different subunit combinations have been presented, which has been proved helpful in understanding the receptors' pharmacology as well as signaling and overall function (Laverty et al., 2017; Masiulis et al., 2019; Miller et al., 2017; Miller & Aricescu, 2014).

The most common $GABA_AR$ subunit composition in the CNS has two α, two β and one μ or another subunit (Wang, 2011). The most abundant subtype in the brain contains two α_1, two β_2, and one γ_2 subunit (McKernan & Whiting, 1996). The general heteropentameric architecture of $GABA_AR$ is represented in Figure 2.

Different $GABA_AR$ subtypes with various subunit combinations have their characteristic subcellular localization, regional specificity and sensitivity to pharmacological agents (Wang, 2011). When it comes to neurosteroids, it has been demonstrated that the β subunit isoform has almost no effect on the neurosteroid modulating actions (Belelli et al., 2002). Receptors containing α_1, α_3 or α_6 are more sensitive to allo-PA modulation compared to those containing α_2, α_4 and α_5 subunits (Belelli et al., 2002). Interestingly, the effect of the δ subunit on neurosteroid sensitivity has been vigorously examined with oftentimes conflicting results, as will be discussed it in the next chapters.

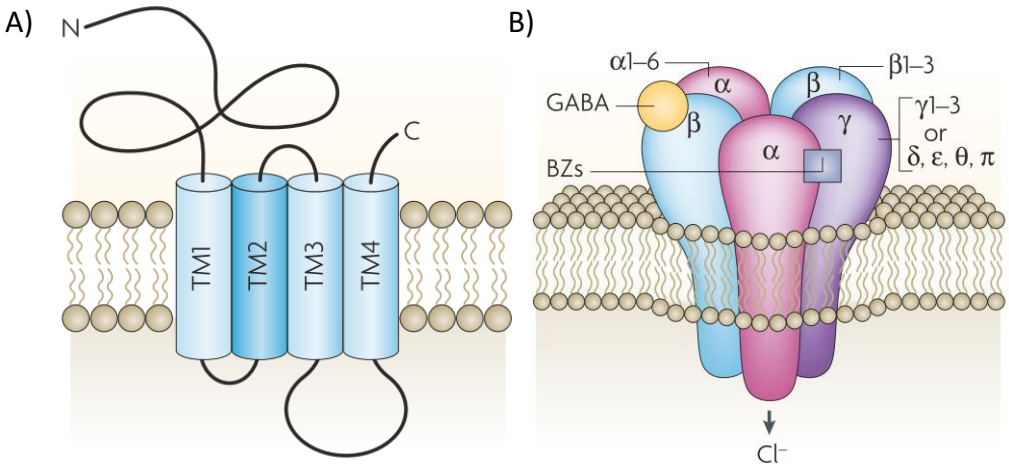

Figure 2: Schematic representation of GABA$_A$R composition. A) One GABA$_A$R subunit has four transmembrane helices (TM1 – TM4), with the TM2 helices forming the channel's central pore, an intracellular domain between TM3 and TM4 (ICD), and an extracellular domain containing both N and C terminals (ECD). **B)** Pentameric GABA$_A$R containing two α, two β and one other distinct subunit, creating together a functional ion channel. GABA and benzodiazepine binding sites on the extracellular domains of the receptor are also depicted. Adapted from (Jacob et al., 2008).

3.2. GABA$_A$R subcellular localization

GABA$_A$R are mainly localized on the postsynaptic neuron, although there are some receptors on the presynaptic axons (Eccles et al., 1963) which account for GABA-mediated presynaptic modulation (Jang et al., 2006). It has been shown that neurosteroids, such as allo-PA, can act via these presynaptic receptors to affect the release of neurotransmitters (Iwata et al., 2013; Kim et al., 2011).

Postsynaptic GABA$_A$R can be divided into synaptic and extrasynaptic receptors. As was reviewed in detail in (Farrant & Nusser, 2005), synaptic and extrasynaptic GABA$_A$R differ in subunit combinations, sensitivity to pharmacological agents and are activated by different GABA concentrations. Most of the synaptic GABA$_A$R contain a γ_2 subunit, together with either α_1, α_2 or α_3 subunit (Chua & Chebib, 2017). These receptors are activated by transiently elevated micromolar concentrations of synaptically released GABA. Activation of synaptic GABA$_A$R results in fast phasic inhibition (Farrant & Nusser, 2005). On the other hand, extrasynaptic GABA$_A$R are mostly responsible for so-called tonic inhibition arising from lower concentrations of ambient GABA and consist of a δ subunit which preferentially assembles with α_6, α_4 and α_5 (Farrant & Nusser, 2005) (Figure 3).

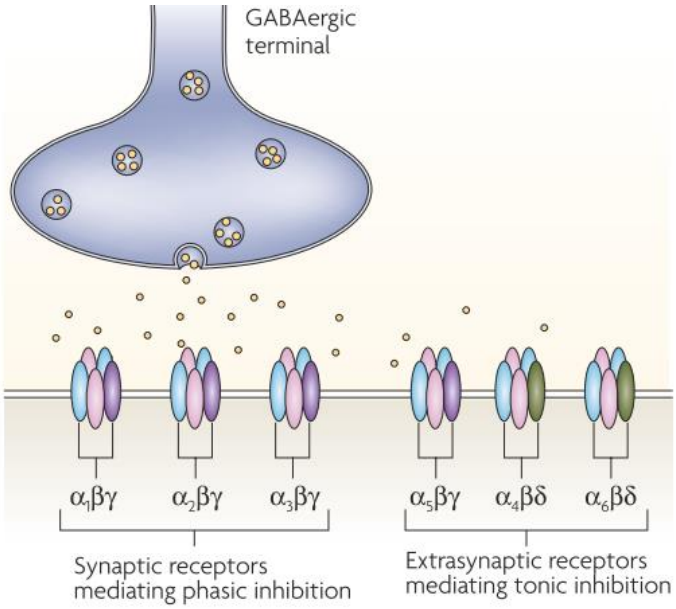

Figure 3: $GABA_AR$ with different subunits generally have a different subcellular localization. For example, the $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_2\beta_{2/3}\gamma_2$, and $\alpha_3\beta_{2/3}\gamma_2$ receptors are mostly located in synapses, while the $\alpha_6\beta_n\delta$, $\alpha_4\beta_n\delta$, $\alpha_1\beta_n\delta$, and $\alpha_5\beta_n\gamma_2$ are mostly extrasynaptically located (Wang, 2011). Adapted from (Jacob et al., 2008).

4. Effects of allo-PA and PA on $GABA_AR$

4.1. Neurosteroid interaction with the $GABA_AR$ complex

It has been over 70 years since Hans Selye demonstrated strong anesthetic effects of several steroidal compounds, including progesterone and deoxycorticosterone acetate, in mice after peritoneal injection (Selye, 1941). Since this discovery, it took more than four decades to characterize the specific effects of progesterone derivatives allo-PA and PA on $GABA_AR$.

4.1.1. Allo-pregnanolone

An important step in this direction was made by Majewska et al. In their studies they found that progesterone and deoxycorticosterone metabolites allo-PA and THDOC respectively are potent modulators of $GABA_AR$ complex (Majewska et al., 1986). The authors showed that, similarly to barbiturates, allo-PA and THDOC inhibit specific binding of a radioactively labeled ligand *t*-butylbicyclophosphorothionate ([^{35}S]TBPS), which typically binds close to the $GABA_AR$ Cl^- channel (Majewska et al., 1986). With another binding study the researchers concluded that these neurosteroids act via an interaction with the $GABA_AR$ (Majewska et al., 1986).

Allo-PA and THDOC increased the influx of radioactively labeled $GABA_AR$-mediated Cl^- ($^{36}Cl^-$) into brain vesicles. (Majewska et al., 1986). Maximal potentiation of the $^{36}Cl^-$ uptake was at 1μM steroid concentration which was diminished by prior incubation with $GABA_AR$ antagonist picrotoxin (Majewska et al., 1986).

In their electrophysiology experiments, the group showed that allo-PA has direct effects on $GABA_AR$-mediated whole-cell responses. Cultured hippocampal rat neurons were recorded in the current-clamp configuration. Cells were depolarized by 100 msec long 0.6 nA pulses. Brief application of 50μM GABA mediated hyperpolarization that caused a delay in the generation of new action potentials. Application of 300nM allo-PA prolonged this action of GABA (Majewska et al., 1986). Under voltage-clamp conditions, 1μM allo-PA reversibly increased the amplitude and the duration of GABA-mediated Cl^- currents and induced an inward current response even in the absence of GABA (Majewska et al., 1986).

4.1.2. **Pregnanolone**

The effects of pregnanolone on GABA-mediated transmission were first described by Harrison et al., 1987 and Callachan et al., 1987. These studies found that PA, similarly to allo-PA, binds to the $GABA_AR$ and enhances its function. In binding experiments analogous to the ones performed in (Majewska et al., 1986), it was shown that PA acts in a similar manner as allo-PA (Harrison et al., 1987). The study identified an important structural feature of neurosteroids active on the $GABA_AR$, specifically the hydroxyl group at carbon C3 in the α position. They observed no major differences between the actions of allo-PA and PA with allo-PA being slightly more potent (Harrison et al., 1987). The higher potency of 5α- over 5β- reduced analogues at $GABA_AR$ has also been reported by (Gee & Lan, 1991; Harrison et al., 1987).

In their electrophysiology experiments Harrison et al., 1987 found that 100nM PA potentiated the amplitude of currents mediated by 10μM GABA and prolonged GABA-evoked responses. The potentiation of evoked current amplitude was dose dependent. At 1μM concentration, the steroid also increased the baseline current even before the application of GABA, which points at direct receptor activation by the neurosteroid (Harrison et al., 1987).

As suggested above, neurosteroids allo-PA and PA show two specific ways of $GABA_AR$ modulation (Wang, 2011): positive allosteric modulation of $GABA_AR$ and direct $GABA_AR$ activation. These two effects are going to be discussed in the next few chapters.

4.2. Positive allosteric modulation of $GABA_AR$

Callachan et al. studied the actions of PA in chromaffin cells from bovine adrenal medulla, where $GABA_AR$ can be also found (Bormann & Clapham, 1985). Under voltage-clamp configuration, 100μM GABA was locally applied using pressure ejection, which resulted in whole-cell currents. In the presence of 100nM PA, these currents were enhanced compared to their control value (Callachan et al., 1987) (Figure 5A). Several concentrations of PA were examined, and a dose-response curve was established (Figure 5B). Responses were visible with PA concentrations as low as 10nM (Callachan et al., 1987). Other studies observed similar results in terms of GABA-induced current potentiation by allo-PA or PA (Peters et al., 1988; Puia et al., 1990).

The group of Callachan et al. tested whether PA acts through the same binding site as another class of $GABA_AR$ modulators, benzodiazepines. Competitive benzodiazepine antagonist Ro 15-1788 had no effect on the steroid-induced potentiation. This experiment eliminated the possibility that PA acts via binding to the benzodiazepine binding site (Callachan, Cottrell, Hather, Lambert, Nooney, et al., 1987). The site and mechanism of action of PA are also different from those of barbiturates, another potent class of $GABA_AR$ modulators, as shown in later studies (Peters et al., 1988).

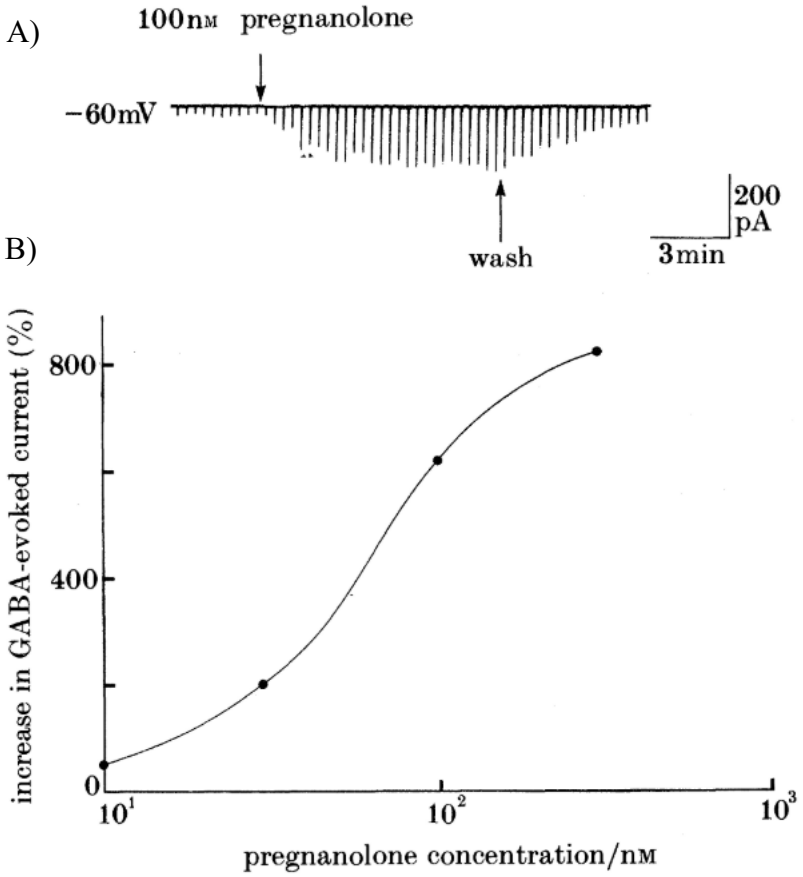

Figure 5: A) In cultured bovine adrenal chromaffin cells, whole-cell responses to 100μM GABA were substantially potentiated by the application of 100nM PA. **B)** Dose-response curve for the potentiation of GABA-evoked currents by PA. Adapted from (Callachan et al., 1987).

It has been also reported that the outcome of PA and allo-PA modulating actions is dependent on GABA concentration (Le Foll et al., 1997). In a study on frog melanotrophs, 1μM allo-PA potentiated the currents evoked by 0.1 – 10μM GABA. In contrast, allo-PA had no effect on responses mediated by maximum effective concentration of GABA (Le Foll et al., 1997). Other studies examining the relationship between the effects of allo-PA and GABA concentration confirmed these observations (Haage & Johansson, 1999).

4.3. Direct activation of $GABA_AR$

Not only do allo-PA and PA act as $GABA_AR$ allosteric modulators, but at higher concentrations they also directly activate the $GABA_AR$. This was demonstrated in the works of Majewska et al., Harrison et al. and Callachan et al.

In cultured hippocampal neurons held at -60mV, brief application of 1μM allo-PA initiated inward Cl^- currents even without the addition of GABA (Majewska et al., 1986).

Harrison et al. saw an increase in the baseline membrane inward current mediated by 1μM PA alone, even before the application of GABA, and the magnitude of this response was dose dependent (Harrison et al., 1987). In another study, bath application of PA at 1μM concentration and higher directly activated $GABA_AR$ and evoked inward currents (Callachan et al., 1987), with a similar reversal potential as those evoked by GABA. These currents could be reversibly blocked by 3μM $GABA_AR$ antagonist bicuculline, whereas phenobarbitone and diazepam reversibly potentiated them (Callachan et al., 1987).

Direct activation of $GABA_AR$ by 1μM and higher allo-PA has also been observed in a study by Puia et al. in human embryonic cells 293 (HEK 293) expressing different human $GABA_AR$ subunits (Puia et al., 1990). Aligning results were obtained by Haage and Johansson with the application of 2μM allo-PA (Haage & Johansson, 1999).

It is interesting that some studies suggest that the direct gating of $GABA_AR$ channel occurs even at neurosteroid concentrations lower than above-mentioned 1μm. Shu et al. showed that application of 300nM allo-PA to neuronal microisland cultures resulted in a slowly developing current which was sensitive to bicuculline (Shu et al., 2004) and that even 100nM allo-PA alone was responsible for significant decrease in neuronal excitability and its ability to develop action potentials (Shu et al., 2004). The authors then hypothesized that this slow receptor activation is due to the neurosteroid accumulation in the neuronal plasma membrane and that steroids access the receptor through this membrane reservoir, which was later supported by other publications (Akk et al., 2005).

4.4. Neurosteroid actions and corresponding $GABA_AR$ binding sites

Positive $GABA_AR$ neurosteroid modulators need a hydroxyl group on the C3 carbon in the α position and a ketone group on the C20 carbon. These groups enable them to bind to the receptor via hydrogen bonds. When testing the effects of amino acid residue mutations and examining the homology with the nicotinic acetylcholine receptor, researchers identified and localized residues that form a part of two specific neurosteroid binding sites mediating the two main mechanisms of neurosteroid action (Hosie et al., 2006). The residues and their respective localizations inside the receptor are represented in Figure 6.

The potentiating site consists of glutamine 241 (Gln 241), asparagine 407 (Asn 407) and tyrosine 410 (Tyr 410) (Hosie et al., 2006). Gln 241 is found inside the α subunit's M1 domain, whereas Asn 407 and Tyr 410 lie in the M4 helix (Hosie et al., 2006).

The amino acid residues that create the binding site responsible for direct activation of the receptor are threonine 236 (Thr 236) and tyrosine 284 (Tyr 284) (Hosie et al., 2006). Thr 236 lies at the M1 transmembrane helix of the α subunit, near the α/β subunit interface and Tyr 284 is in the M3 domain of the β subunit (Hosie et al., 2006).

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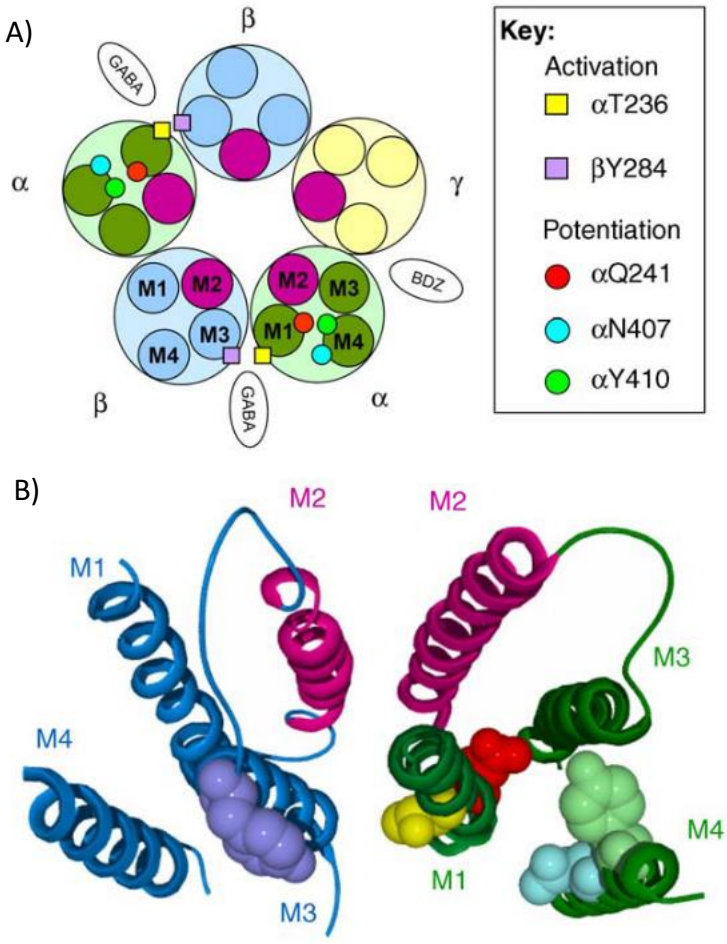


Figure 6: Schematic representation of neurosteroid sites of action on $GABA_AR$. A) Representation of heteropentameric $GABA_AR$, each of the subunits with four transmembrane helices (M1 – M4). The site for potentiating actions of neurosteroids lies inside the transmembrane domain of α subunits, whereas the site mediating a direct activation of the receptor is placed on the interface of α and β subunits. Amino acid residues forming the binding sites are indicated in the representation, as well as in the table on the right. GABA and benzodiazepine binding sites

are also shown. **B)** Neurosteroid binding sites in a ribbon model of α and β $GABA_AR$ subunits, color-coded corresponding to the first picture. Figure adapted and modified from (Chisari et al., 2010).

A new look at the binding sites for potentiating neurosteroids has been proposed in a recent paper (Laverty et al., 2017). By creating a novel chimeric $GABA_AR$, the researchers obtained a precise location of the neurosteroid binding site. They argued that rather than having two separate sites for the two specific neurosteroid actions, direct activation and potentiation of $GABA_AR$ both arise from neurosteroid interacting with a single interfacial binding site (Laverty et al., 2017). Other studies keep proposing new previously unidentified neurosteroid binding sites on the $GABA_AR$ (Chen et al., 2018). Hence, the discussion regarding the neurosteroid sites of action remains ongoing.

5. Neurosteroid effects on neuronal activity

$GABA_AR$ are responsible for two types of neuronal inhibition. On one hand, synaptic receptors are activated in a phasic manner by synaptically released high concentrations of GABA, which causes inhibitory postsynaptic currents and potentials (IPSCs and IPSPs) and the hyperpolarization of the postsynaptic membrane. On the other hand, extrasynaptic $GABA_AR$, localized outside the synaptic cleft and activated either by spilled-over GABA from the synapse or by the low concentration of extracellularly present neurotransmitter, create tonic inhibitory membrane current (Farrant & Nusser, 2005).

Neurosteroids modulate both types of $GABA_AR$-mediated inhibition (Figure 7). However, it is still not completely clear what are the preferential steroid targets, exactly how steroid sensitivity varies for different types of receptors, or what specific neurosteroid actions account for the behavioral and clinical outcomes of the steroid modulation. In this chapter, we will discuss the role of $GABA_AR$ subunit composition, receptor subcellular localization and the type of $GABA_AR$ activation on the effect of neurosteroids on neuronal activity.

5.1. Neurosteroid actions at synaptic and extrasynaptic receptors

$GABA_AR$-potentiating neurosteroids modulate the function of synaptic $GABA_AR$ and the properties of IPSCs. In the study which used dissociated hypothalamic rat neurons, allo-PA did not significantly modulate the amplitude of miniature IPSCs (mIPSCs), inhibitory postsynaptic currents evoked by the spontaneous release of neurotransmitter from the presynaptic neuron, without the presence of action potential

(Haage & Johansson, 1999). However, it prolonged the mIPSC decay and increase the mIPSCs frequency, which in addition points to a presynaptic mechanism of action (Haage & Johansson, 1999). The effects of neurosteroids on IPSCs also significantly vary throughout the brain regions and different neuronal subtypes, as reviewed in (Herd et al., 2007).

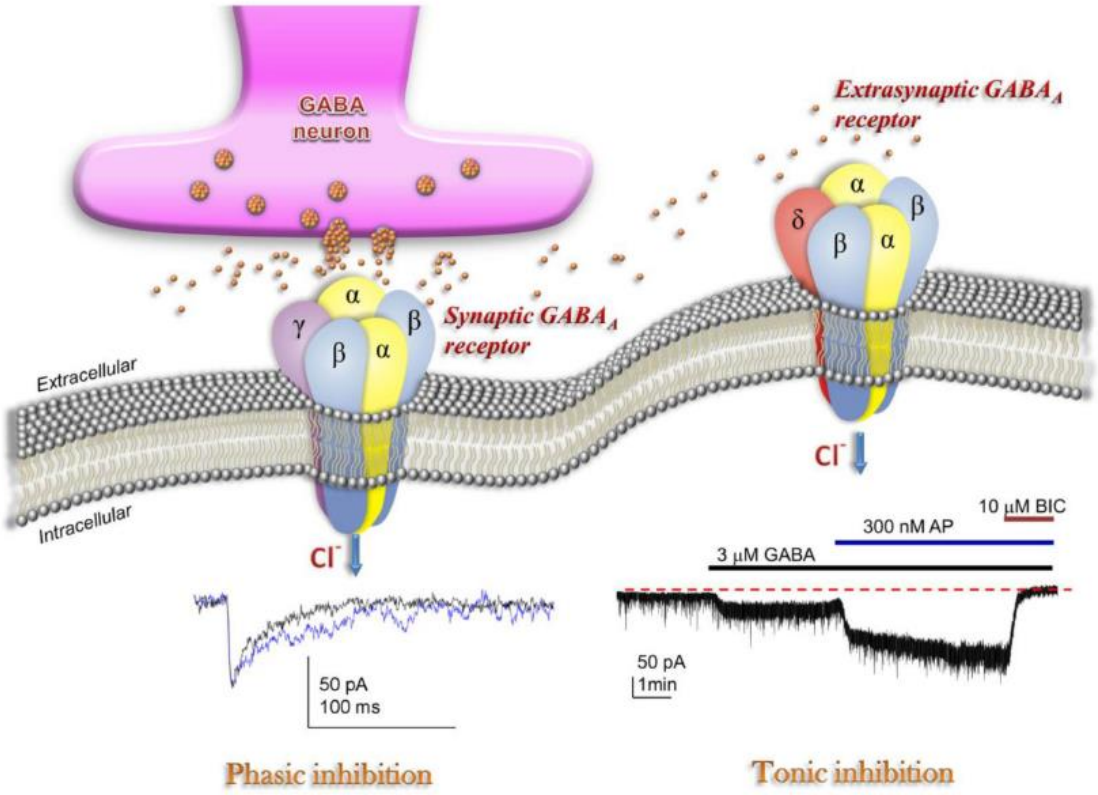


Figure 7: Neurosteroids modulate both synaptic and extrasynaptic GABAAR. Synaptic receptors mediate the fast phasic neuronal inhibition and are activated by transiently high concentrations of synaptically released GABA (top left). Neurosteroids potentiate these receptors by prolonging the deactivation kinetics of inhibitory postsynaptic currents (bottom left). Extrasynaptic receptors are activated by lower concentrations of GABA and they mediate the longer tonic neuronal inhibition (top right). Neurosteroid potentiation of extrasynaptic receptors results in enhancement of baseline currents. Application of 300nM allo-PA (here AP) dramatically increases tonic GABA-mediated conductance, which is diminished by the $GABA_AR$ antagonist bicuculline (bottom right). The neurosteroid modulation of phasic and tonic responses combined create together the total effect on network activity. Adapted from (Carver & Reddy, 2013).

The effects of neurosteroids on tonic $GABA_AR$ currents were demonstrated in the early studies by Majewska et al., Callachan et al. and Harrison et al. (Callachan et al., 1987; Majewska et al., 1986). In some brain regions, such as the

hippocampal dentate gyrus, the extrasynaptic $GABA_AR$ appears to be more sensitive to neurosteroid actions than the synaptic receptors (Herd et al., 2007).

In a study carried out on *Xenopus laevis* oocytes expressing different isoforms of the $GABA_AR$ subunits, incorporation of δ subunit significantly increased the $GABA_AR$-enhancing actions of allo-PA (Belelli et al., 2002). This aligns with other studies exploring the effects of THDOC (Wohlfarth et al., 2002). Another study by Stell et al. supported the idea of preferential actions of neurosteroids on extrasynaptic δ-$GABA_AR$ (Stell et al., 2003). Application of 10nM THDOC, which is similar to its physiological *in vivo* concentrations, significantly potentiated tonic currents in dentate gyrus granule cells and cerebellar cells, but failed to have any significant effect on the synaptic events (Stell et al., 2003). At higher (100nM - 1μM) concentrations, the neurosteroid started to have an effect even on the IPSCs. THDOC not only potentiated the tonic currents, but also prolonged the decay of spontaneous IPSCs (sIPSCs), which are postsynaptic currents mediated by the neurotransmitter released after one action potential (Stell et al., 2003) (Figure 8). Moreover, in a model of δ knockout mice the researchers observed a significant decrease in the $GABA_AR$-mediated tonic currents. These results combined reinforced the idea that δ-$GABA_AR$, preferentially activated by low concentrations of ambient GABA, are responsible for tonic neuronal inhibition, and that this type of $GABA_AR$-function is highly sensitive to neurosteroids. Other papers came to similar conclusions (Carver & Reddy, 2016; Marowsky & Vogt, 2014).

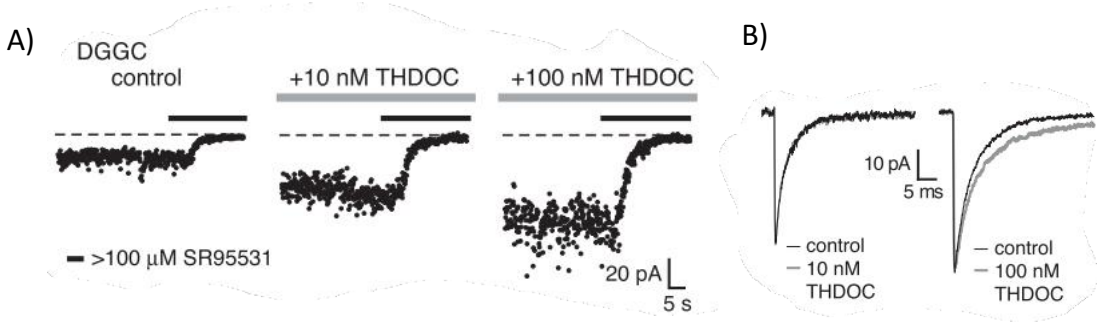


Figure 8: The $GABA_AR$-potentiating neurosteroid THDOC preferentially enhances tonic currents. A) Compared to control, application of 10 and 100nM THDOC potentiated the $GABA_AR$ tonic currents in the dentate gyrus granule cells (DGGC), which was diminished after the application of $GABA_AR$ inhibitor gabazine (SR95531). The level of current potentiation was dose dependent. **B)** 10nM THDOC had no effect on the amplitude and the decay of sIPSCs, whereas 100nM THDOC prolonged the decay of sIPSCs in DGGC. Adapted and modified from (Stell et al., 2003).

However, the literature has not been completely unanimous. Mihalek et al. showed not only a substantially decreased sensitivity of δ knockout mice to pregnanolone (Mihalek et al., 1999), but also a change in the dynamics of synaptic responses, such as faster decay of mIPSCs compared to control animals (Mihalek et al., 1999). Similar results were also shown in (Spigelman et al., 2003), where the authors observed faster decay of not only mIPSCs, but also of evoked IPSCs, suggesting the presence of δ-containing receptors both at the synapses and outside of them. In an earlier study by Zhu et al., the δ-containing $GABA_AR$ were much less sensitive to modulatory actions of THDOC (Zhu et al., 1996), which is in contrast to most of the results presented so far.

5.2. The unclear roles of $GABA_AR$ δ and γ_2 subunits

To date, there have been many reports suggesting the preferential effect of $GABA_AR$-potentiating neurosteroids on extrasynaptic receptors and tonic inhibition. However, the participation of δ and γ_2-containing $GABA_AR$ in the different types of neuronal inhibition, as well as the δ-$GABA_AR$ and γ_2-$GABA_AR$ sensitivity to neurosteroids remain yet to be fully explained. Recent papers by the groups of Gustav Akk and Steven J. Mennerick have demonstrated that the distinction between synaptic vs. extrasynaptic receptors and the tonic vs. phasic inhibition mediated by them is non-trivial.

Since it can be often challenging to study the δ-containing $GABA_AR$ in native cells, Shu et al. created a fused α_4/δ subunit, to ensure co-expression of the two subunits as seen in physiological conditions and to study the pharmacological properties of the α_4/δ-$GABA_AR$ compared to other receptor subunit combinations (Shu et al., 2012). In their experiments on *Xenopus laevis* oocytes, they found no qualitative differences between the extrasynaptic like α_4/δ-$GABA_AR$ and the synaptic-like $\alpha_1\beta_1\gamma_2$L-$GABA_AR$ in terms of neurosteroid potentiation. Maximum potentiation by THDOC was higher at the synaptic-like receptors. 30nM - 100nM allo-PA resulted in potentiation of both of the receptor subtypes (Shu et al., 2012). The most noticeable difference in the neurosteroid potentiation was apparent with high concentrations of applied GABA. While the α_4/δ-$GABA_AR$ were significantly potentiated by 1μM all-PA, the $\alpha_1\beta_1\gamma_2$L-$GABA_AR$ were not affected by the neurosteroid. The authors argue that this effect is due to GABA acting as a partial agonist on the extrasynaptic-like receptors, while having a higher efficacy at the synaptic-like receptors (Shu et al., 2012).

$\alpha_1\beta_1\gamma_2$L-GABA$_A$R have been also shown to produce persistent tonic currents in the presence of 0,5μM ambient GABA, with and without the application of the synthetic neurosteroid alphaxalone (Li & Akk, 2015). Hence, tonically activated synaptic GABA$_A$R might account for some of the clinical effects of GABA$_A$R modulators (Germann et al., 2019; Li & Akk, 2015). Conversely, δ-GABA$_A$R were proposed to be activated by both synaptically released GABA, as well as the GABA that diffuses from the synapse. It was suggested that synaptic events can be in part evoked even at δ-containing receptors (Sun et al., 2018, 2020). Finally, another study found no selectivity of allo-PA actions at δ-containing and non-δ-containing GABA$_A$R (Lu et al., 2020). Using a transgenic mouse model with introduced picrotoxin resistance associated with either the δ- or γ_2-containing receptors, the researchers showed that allo-PA potentiated both phasic inhibition, mainly via γ_2-GABA$_A$R, and tonic inhibition, mediated by both types of receptors (Lu et al., 2020).

6. Clinical relevance of GABA$_A$R-potentiating neurosteroids

6.1. Behavioral effects of allo-PA and PA

Allo-PA and PA, as well as other GABA$_A$R-potentiating neurosteroids, exhibit several actions in *in vivo* systems, which include anxiolytic, anti-convulsant and anesthetic effects (Mingde Wang, 2011). The anesthetic effect of steroids has been first shown by (Selye, 1941). Another one of the neurosteroid functions is their involvement in the stress response. In the later decades, it was demonstrated that the brain and plasma allo-PA concentrations rapidly and significantly increase in acute stressful conditions (Purdy et al., 1991). An acute depletion of GABA in handling-habituated rats resulted in an increase of brain and plasma levels of allo-PA, which was also observed in animals that underwent two types of stress paradigms leading to the decrease of GABA$_A$R-mediated transmission (Barbaccia et al., 1997). However, during chronic stress there has been observed conversely a decrease in the levels of neurosteroids, as well as of GABA$_A$R-mediated transmission (Serra et al., 2000). These results suggest a protective role of GABA-mediated neurotransmission and its modulation by neurosteroids in the stress response (Zorumski et al., 2013).

In rodent studies, allo-PA and PA were proven to have anxiolytic effects, consistent with their role as positive GABA$_A$R modulators (Bitran et al., 1991, 1995, 1999). Animals after intrahippocampal injections of allo-PA were more explorative, as demonstrated by a

higher number of entries and the time spent in the open arm of the elevated plus maze compared to controls (Mòdol et al., 2011). Other studies have shown that manipulation of the neurosteroid levels in neonatal rats influences hippocampal development and shapes animal behavior displayed during adolescence and adulthood (Darbra et al., 2013; Mukai et al., 2008).

6.2. Allo-PA and PA in health and disease

The clinical importance of allo-PA and PA-mediated modulation is represented by their involvement in pregnancy and the regulation of mood. The imbalance of the neurosteroid levels in the brain has been connected to symptoms of postpartum depression (PPD), posttraumatic disorder (PTSD) and major depressive disorder (MDD) (Maguire, 2019; Wang, 2011).

During the menstrual cycle of females, the levels of allo-PA and PA correspond to the changing levels of circulating progesterone (Wang et al., 1996) and they are in the range of low nM concentrations, with the highest concentrations found in the luteal phase of the cycle (Genazzani et al., 1998; Wang et al., 1996). However, during the pregnancy period, the levels progesterone and estrogen, as well as the levels of allo-PA and PA, increase dramatically. At the third trimester, the concentration of plasma allo-PA reaches up to 50nM and the concentration of PA up to 25nM (Hill et al., 2007). $GABA_AR$-mediated transmission is then downregulated as a compensation to prevent anesthesia (Mody, 2019). The levels of neurosteroids drastically drop post-partum and this imbalance between the attenuated $GABA_AR$ neurotransmission and neurosteroid concentration is believed to lead to the symptoms of PPD (Mody, 2019). In 2019, allo-PA was approved by the American Food and Drug Administration as a therapeutical drug for the treatment of moderate to severe PPD (Meltzer-Brody et al., 2018; Mody, 2019). In a double-blind randomized study, patients who received a single allo-PA injection showed a significant decrease of the Hamilton Rating Scale for Depression (HAM-D) score, with fast-onset and durable responses (Meltzer-Brody et al., 2018). It is therefore essential to understand the complexity of $GABA_AR$-modulating neurosteroid actions in *in vitro* neuronal networks and *in vivo* systems to further utilize these compounds in clinical context.

7. Goals of this work

The effects of $GABA_AR$-potentiating neurosteroids on neuronal activity have been extensively studied in molecular studies, electrophysiology experiments with relatively short applications of neurosteroids to individual cultured neurons, and on a wide range of time scales, from hours to days in behavioral experiments. Even longer neurosteroid effects on the organism have been studied in the works examining the levels of neurosteroids in pregnancy, menstrual cycle and neurological disorders, culminating in the approval of allo-PA as a treatment for postpartum depression. There is however still a lack of understanding of the dynamics of the influence of $GABA_AR$-potentiating neurosteroids on neuronal networks in the context of overall activity, given their multiple effects on different types of receptors and dynamic metabolism. In this work we aim to establish a calcium imaging technique using a genetically encoded calcium indicator to monitor neuronal network activity *in vitro.* Our efforts could eventually lead to the use of the calcium imaging method to study prolonged or repeated applications of the neurosteroids.

The objectives of this work are:

1) Establish an experimental setup with a solution application system for calcium imaging of primary neuronal cultures, using the jGCaMP7f calcium indicator.

2) Validate the system by testing the effect of tetrodotoxin on the observed culture activity.

3) Test the effects of the neurosteroid pregnanolone on spontaneous network activity of primary hippocampal cultures by optically measuring the changes in intracellular concentration of Ca^{2+} ions, which will be represented by the change of fluorescence of the jGCaMP7f protein. Compare the gained results with electrophysiology current-clamp recordings.

4) Examine the effects of pregnanolone on neuronal activity after repeated applications.

8. Material and methods

8.1. Chemicals

All the chemicals used were supplied by Sigma and Serva, the components for cell culture media were either from Sigma or Gibco. The water used for the preparation of solutions was deionized with a Milipore Simplicity 185 purification system. The solutions were sterilized by filtration. TTX was purchased from Tocris, PA was provided by the group of Dr. Eva Kudova at the Institute of Organic Chemistry and Biochemistry, CAS.

8.2. Primary hippocampal mass cultures

Primary astrocyte and hippocampal cultures were prepared according to standard protocols. Briefly, primary cortical astrocytes were isolated from newborn male Wistar rat pups at postnatal day 0 – 2 (P0 – P2). Dissected cortices were incubated for 15 min at 37°C in 0.05% trypsin-EDTA solution (Gibco). After incubation, trypsin was deactivated, and cortices dissociated by gently triturating with a micropipette tip. 50 µl of cell suspension per 75 cm^2 flask was transferred into flasks filled with astrocyte medium. Cells were incubated in 37°C and 5% CO_2 for 12 days in a medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX (Gibco), HEPES (Gibco), 10% fetal bovine serum (FBS) (Gibco) and penicillin/streptomycin (pen/strep) (Sigma).

After 12 days of incubation, astrocytes were plated into 35mm petri dishes containing 4 – 5 glass coverslips (10mm diameter, thickness #0), precoated with poly-D-lysine (Sigma) and collagen (Serva). The estimated cell density was around 6000 cells/cm^2 (60 000 cells/dish). Cells were allowed to grow for 2 days in 37°C and 5% CO_2, until they reached confluency. Right before plating neurons, astrocyte growth medium was completely replaced with pre-warmed neuronal medium.

Hippocampi were dissected from P0 – P2 Wistar rat pups and incubated in papain solution (Worthington Biochemical Corp.) for 45 min in 37°C and 5% CO_2. Afterwards, the hippocampi were washed and placed into prewarmed neuronal growth medium, which consisted of Neurobasal A, GlutaMAX, vitamin B27 supplement (all Gibco) and pen/strep (Sigma). The hippocampi were mechanically dissociated similarly to the cortices, as described above. Finally, neurons were plated to petri dishes on top of the already grown astrocyte feeder layer, 400 000 – 500 000 cells/dish. Cultures were then incubated in 37°C and 5% CO_2 for 3 – 4 days.

At 3 - 4 days *in vitro* (DIV), the hippocampal mass cultures were treated with 5µM β-D-arabinofuranosid (AraC) (Sigma) for 24 hours to stop astrocyte proliferation.

8.3. Viral transduction

Premade AAV9 (adeno-associated virus 9) particles were used for the expression of the jGCaMP7f calcium indicator. Specific sensor expression in neurons, but not in glial cells, was achieved due to the synapsin promotor. Viral particles were produced from pGP-AAV-syn-jGCaMP7f-WPRE plasmid and obtained in a ready-to-use form from Adgene. Stock solution was divided into 10µl aliquots, with 0.5µl of stock solution (titer $2.6 \cdot 10^{13}$ GC/ml) and 9,5µl of extracellular solution (ECS) and stored at -80°C until the day of transduction.

The extracellular solution used for creating the virus aliquots and during the calcium imaging experiments consisted of 160mM NaCl, 2.5mM KCl, 10mM glucose, 10mM HEPES, 1mM $MgCl_2$ and 2mM $CaCl_2$, all dissolved in deionized water and sterilized using filtration.

Transduction was performed 4 – 5 days after the plating of the primary hippocampal cultures. One 10µl aliquot of viral solution was added per 2ml of neuronal medium in one well, with a final concentration of the virus being about $2.6 \cdot 10^9$ GC/ml.

Cultures were then allowed to incubate at 37°C and 5% CO_2 for 7 – 8 days, until the start of live-cell calcium imaging experiments. The transduced cell cultures were handled according to appropriate biosafety level standards.

8.4. Calcium imaging of the primary hippocampal culture activity

8.4.1 The jGCaMP7f sensor

For the calcium imaging of the primary rat hippocampal cultures we chose a genetically encoded calcium indicator jGCaMP7 (GCaMP7 developed at the Janelia Research Campus) (Dana et al., 2019). We specifically used the jGCaMP7f variant with fast kinetics, which was designed for the tracking of dynamic changes in neuronal activity of individual cells (Dana et al., 2019). GCaMP is one of the genetically encoded calcium indicators (Grienberger & Konnerth, 2012) and is commonly used to measure the activity of neural systems, both *in vivo* and *in vitro* (Nakai et al., 2001). It consists of a circularly permuted green fluorescent protein, calmodulin attached to its N-terminus and a calmodulin-binding

peptide connected to the GFP C-terminus (Figure 9) (Nakai et al., 2001). Compared to the classical chemical fluorescent calcium indicators, some of the main strong features of the genetically encoded sensor are its fast kinetics, high sensitivity and the exclusive expression in neurons but not in glial cells (Dana et al., 2019).

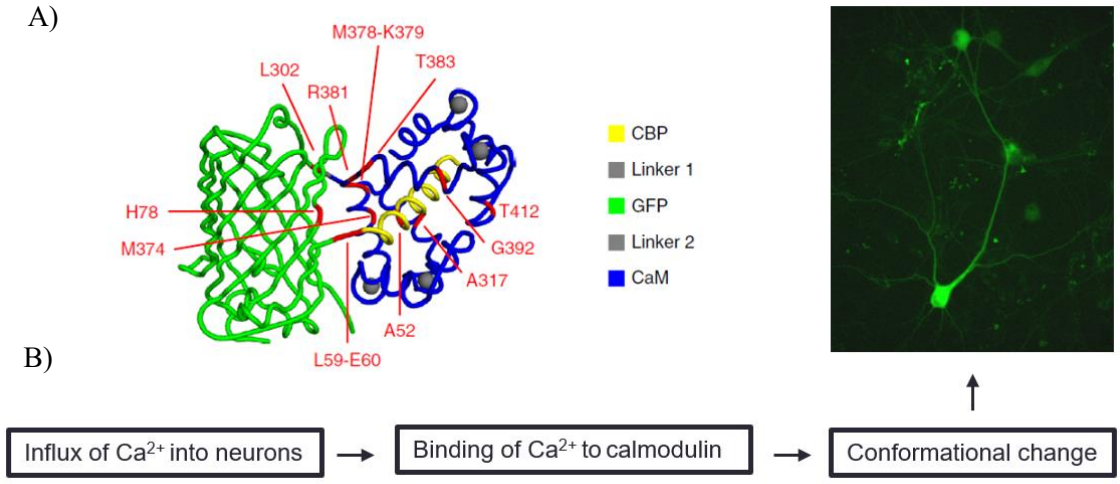


Figure 9: Schematic representation of the cytoplasmatic jGCaMP7 sensor and its mechanism of function. A) Molecular structure of the jGCaMP7 variant, consisting of a green fluorescent protein (GFP) (green), calmodulin (CaM) (blue), calmodulin-binding peptide (CBP) (yellow), linker peptides and four Ca^{2+} ions (grey) bound to CaM. Mutated areas that were tested while developing the different jGCaMP7 variants are depicted in red. **B)** During neuronal activity there is a major influx of Ca^{2+} into the postsynaptic neuron. These free ions can then bind to the calmodulin which results in a conformational change of the fluorophore and ends with an increase of the sensor's fluorescence. The image of jGCaMP7 structure was adapted from (Dana et al., 2019).

8.4.2 Live-cell fluorescent microscopy experiments

Calcium imaging experiments were performed with the hippocampal neuronal cultures at 12 – 14 DIV. Coverslips with hippocampal mass cultures were broken into smaller pieces, washed with prewarmed ECS and transferred one by one into a 35mm petri dish with thin #0 glass bottom, already prefilled with 2ml of warm ECS. The experimental recording was then conducted with a constant flow of solutions through the petri dish. During the experiments, the cells were placed in a heated chamber. The applied solutions were prewarmed in an incubator to 37°C and the application tube was heated throughout the recording.

8.4.3. Application system

Precise application of solutions was achieved with a custom-made application system. This system allowed us to change between up to six different solutions with one common outlet tube (0.5mm diameter). During the experiment, the solutions were allowed to perfuse the petri dish at a speed of about 2 ml/min The removal of the solutions was then mediated by another tube at the opposite side of the dish. When characterizing the experimental setup, we ensured the whole piece of the coverslip was washed over with the flowing solution. Before the start of the experiment, the application tube was positioned right at the tip of the coverslip shard at the height of around 0.5mm from the floor of the dish. The appropriate height and position of the application tube was visually checked before the beginning of every imaging experiment to make sure all the cells were under the constant flow of solution.

8.4.4 Solutions

Standard extracellular solution with Mg^{2+} was used as the baseline experimental solution. PA and control solutions were always prepared fresh before the start of the imaging experiments. PA was first dissolved in dimethyl sulfoxide (DMSO) (Sigma) and then diluted to the appropriate concentration into premade ECS, with the final concentration of the DMSO 0.3%. As a control solution we used ECS with 0.3% DMSO. The TTX was dissolved in deionized water and kept at -18°C. The application solutions were prewarmed to 37°C in the incubator prior to the experiment and were kept warm throughout the recording.

8.4.5 Image acquisition

The active cells expressing the GCaMP fluorescent protein were selected visually. The changes of intracellular Ca^{2+} ion concentration accompanying the spontaneous neuronal activity were recorded as short-term increases of fluorescence intensity of the GCaMP sensor. Live-cell imaging was executed using a Leica DMi8 inverted fluorescence widefield microscope with a 20x objective. A sCMOS camera Photometrics Prime 95B and the LAS X program were used for image acquisition. LED diodes set to 20% intensity and 483 nm wavelength were used for the excitation of the jGCaMP7f protein sensor. The final exposure time was 50 - 55 msec (frequency of acquisition: around 20 frames per sec).

8.5 Result analysis and statistics

After the finished recording, regions of interest (ROIs) that encompassed the bodies of the recorded neurons were selected. The data from these ROIs, including the mean fluorescence of the selected area, were then exported from the LAS X program to Microsoft Excel for further analysis. The background fluorescence intensity of a place with visibly no active neurons was subtracted from the fluorescence intensity of the neuronal bodies.

Analysis of the calcium imaging recordings was done using the Clampfit 10.7 program. We approached the result analysis according to other works that characterized the GCaMP properties in *in vitro* systems (Dana et al., 2019; Wardill et al., 2013). We chose to evaluate several factors representing neuronal activity: amplitude of Ca^{2+} peaks, frequency of Ca^{2+} peaks and area under curve (AUC) of individual peaks and of one-minute intervals. We then mainly focused on peak frequency and amplitude. Peak amplitude was established as the change in fluorescence intensity over basal fluorescence (ΔF/F) (Dana et al., 2019), peak frequency as the number of peaks per minute. To better understand the observed activity of our neuronal cultures, we consulted the literature and tried to roughly estimate the number of action potentials behind individual calcium peaks (Dana et al., 2019; Wardill et al., 2013) (Figure 10). In the future, a more sophisticated method and software would be needed to precisely characterize the patterns of the electrical activity represented by the calcium peaks.

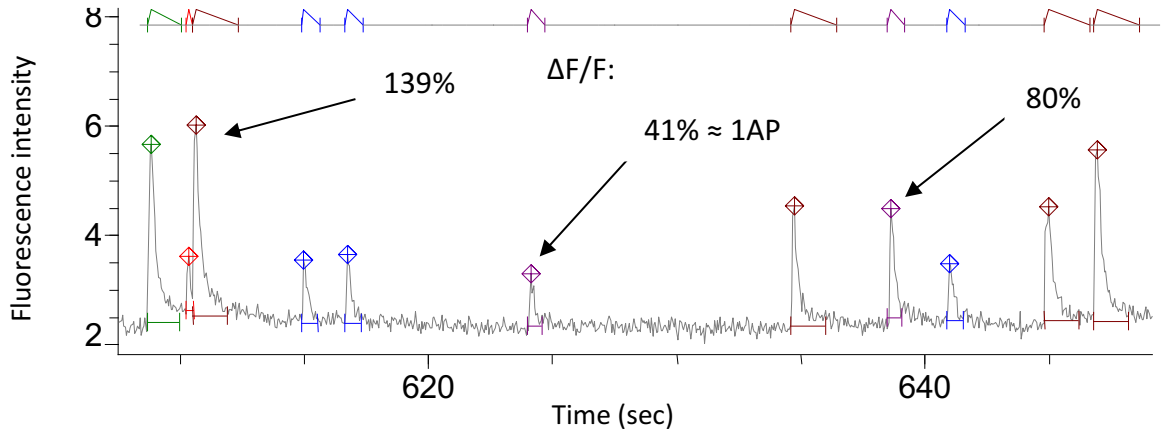


Figure 10: Example of a section of a recording trace from the result analysis conducted in the Clampfit program. Several templates were created to account for the variable amplitude and width of the calcium peaks. By consulting the literature (Dana et al., 2019; Wardill et al., 2013),

we were able to roughly estimate the number of action potentials in one calcium peak, depending on its amplitude and shape.

To account for the variable peak amplitude and shape, we created several different peak templates for each cell. The calcium peaks were then quantified and their characteristics were measured automatically. Peaks that lasted for longer than 2 sec or events that could not be distinguished as individual peaks were rare and were not accounted for in further analysis. For most of the recordings, two of the active cells in the imaged area were selected and further analyzed. Mean values of the peak frequency, amplitude and AUC were calculated for 1 min intervals for each cell. Values of each parameter were then averaged for the two cells from the same recording and these values were taken as n = 1; n value thus represents the number of independent recordings.

Statistical analysis was performed using the Sigmaplot program. We compared the mean values of the calcium peak parameters before, during, and after the application of TTX or the neurosteroid. In the experiments with two subsequent applications of PA we compared the level of inhibition between the two applications. Paired t-test was used to assign statistical significance. The results were considered statistically significant with a P-value $\leq 0{,}05$.

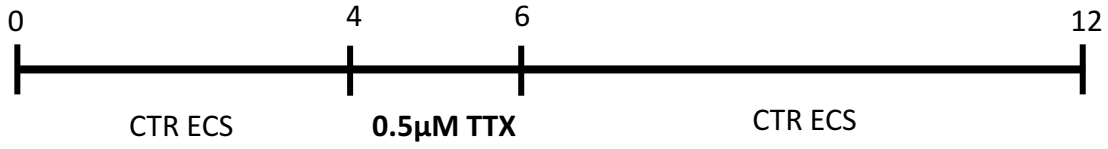
9. Results

9.1. Application of 0.5µM tetrodotoxin inhibits spontaneous calcium peaks.

First, we aimed to validate our newly constructed application system and to verify that the observed changes in fluorescence of the neuronal bodies can be truly attributed to the changes of intracellular Ca^{2+} connected to neuronal activity. To achieve that we used tetrodotoxin (TTX) which is a compound widely used in electrophysiology, pharmacology and various types of live-cell imaging experiments. TTX blocks the sodium voltage-gated channels and inhibits the production of action potentials (Narahashi et al., 1964).

In our experiments, 2 min application of 0.5µM TTX in ECS resulted in a complete extinction of the observed spontaneous peaks (Figure 11). This way, we confirmed that the observed GCaMP fluorescence peaks representing the changes in intracellular are a result of neuronal firing. We also established that the washout system is sufficient for the neurons to return to their basal activity after several minutes of ECS perfusion.

Application timeline:



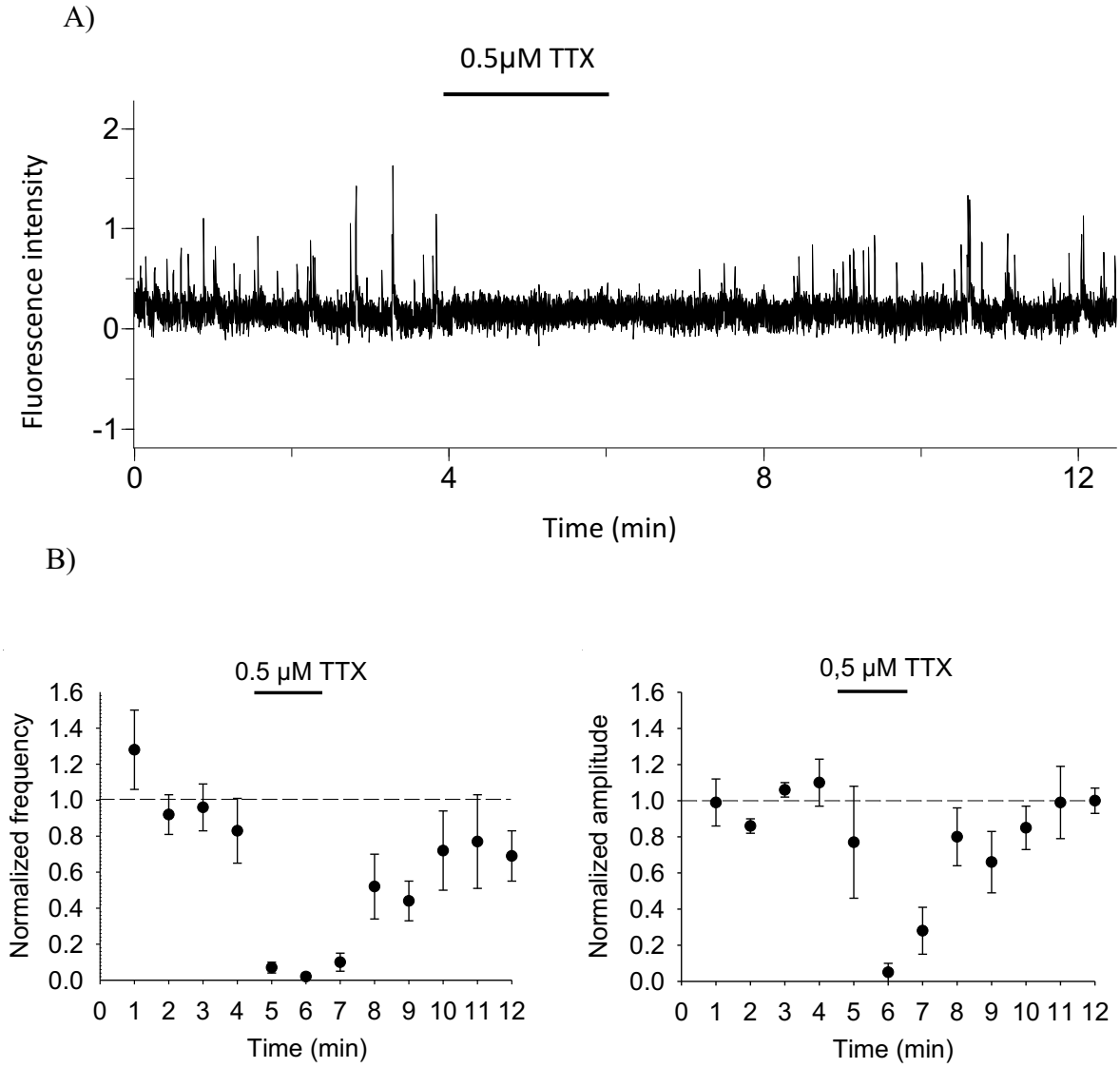


Figure 11: Application of 0.5µM TTX inhibits calcium peaks. A) Example trace of a recording of one cell before, during and after the application of TTX. **B)** Application of 0.5µM TTX fully blocked the calcium peak frequency and amplitude, which gradually returned to their baseline level during the washout period (n = 8; values normalized to the mean value of the first 4 min of baseline activity). Total length of the recording was 12 min, with 4 min application of CTR ECS, 2 min 0.5µM TTX and then 6 min of washout with CTR ECS.

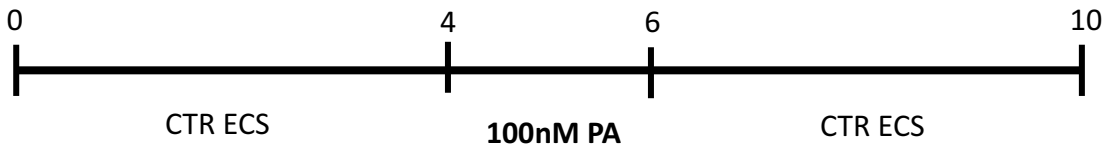
9.2. Inhibition of calcium peaks by 100nM PA is comparable to that seen in current-clamp experiments.

We then planned to examine the effects of PA on spontaneous neuronal network activity. To further validate the custom-made application system and the GCaMP calcium imaging method as reliable tools for measuring spontaneous neuronal activity in our experimental conditions, we compared the effects of 2 min applications of 100nM PA in two different types of recordings: patch-clamp electrophysiology and the GCaMP calcium imaging.

The sample traces in Figure 12 show that application of 100nM PA substantially inhibited both the frequency of action potentials (AP), recorded in electrophysiology experiments (Figure 12A), and the frequency of calcium peaks (Figure 12B). After the return to CTR solution, the APs and calcium peaks gradually returned to their control levels. The onset of the inhibition of the neuronal activity was similar for both types of experiments, however, the washout of the neurosteroid took longer in the calcium imaging experiment. This could be due to some technical differences between the two application systems used for the recordings.

In our experimental conditions, the PA potentiating effects on the $GABA_AR$ manifest themselves as a reduction of spontaneous network activity of the hippocampal cultures. These results prove that the GCaMP calcium imaging system is a valid method for testing the effects of neurosteroids and possibly other pharmacological agents on the neuronal networks *in vitro*. This method can be further used as an additional tool to the electrophysiology experiments studying the patterns of neuronal activity.

Application timeline (min):



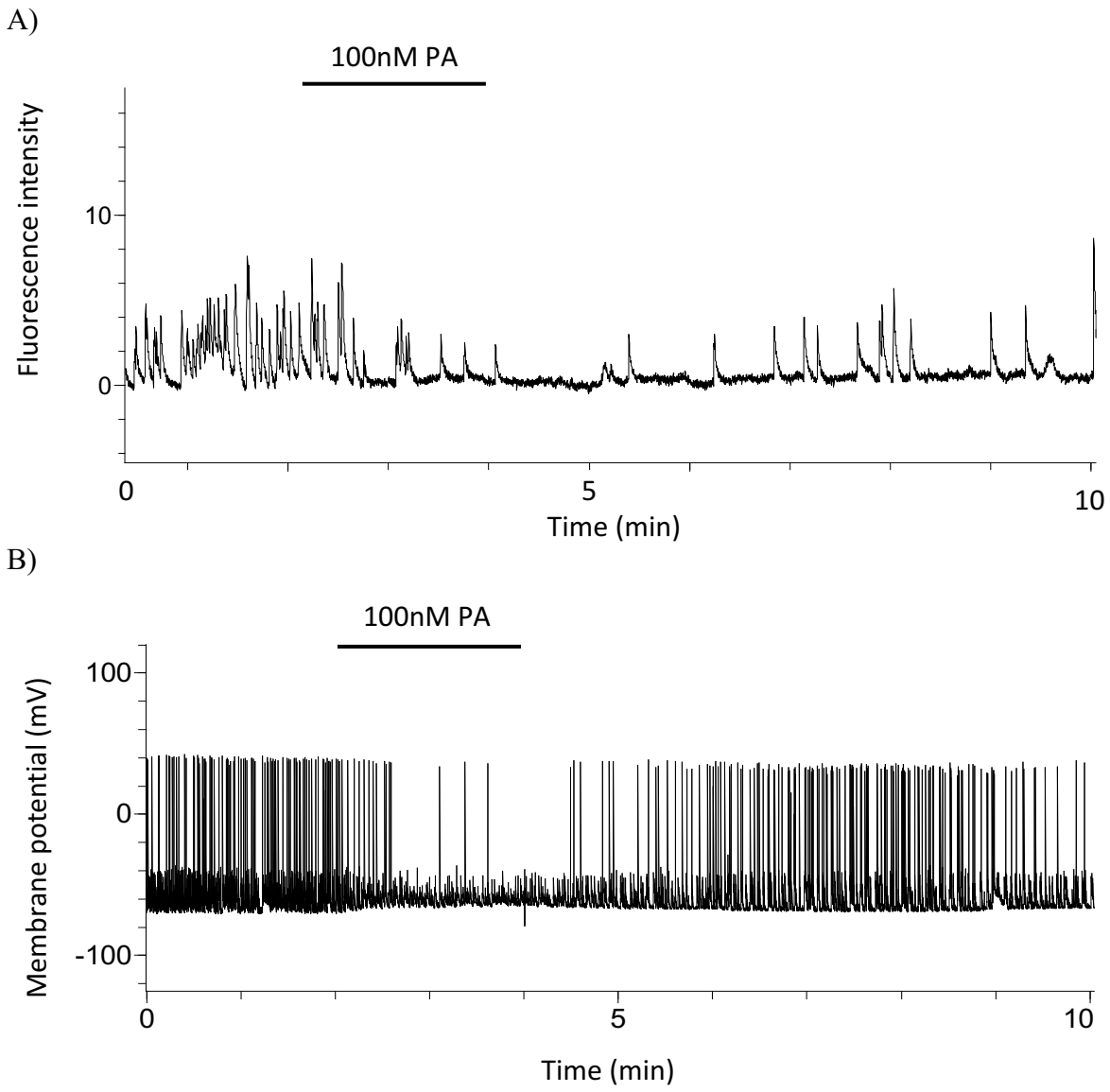


Figure 12: Comparison of two types of recordings examining the effect 100nM PA. Example traces from two different cells recorded with two methods of detecting neuronal activity: A) calcium imaging using the jGCaMP7f sensor and B) patch-clamp recording in the current-clamp configuration. Total length of both of the recordings was 10 min, with 2 min initial CTR ECS application, then 2 min 100nM PA and again CTR ECS for 4 min. In the patch-clamp recording we can see two types of postsynaptic electrical events: subthreshold excitatory postsynaptic potentials (EPSPs) (shorter lines) and action potentials (longer lines). Application of 100nM PA resulted in a substantial inhibition of action potentials.

9.3. 1 min application of 100nM PA decreases the calcium peak frequency compared to control.

Our goal for the next experiments was to determine if the neurosteroid effects on network activity are reproducible during repeated applications or if there is perhaps an adaptation in the network dynamics as a result of the first neurosteroid exposure, which would then result in a change of inhibition after the second application.

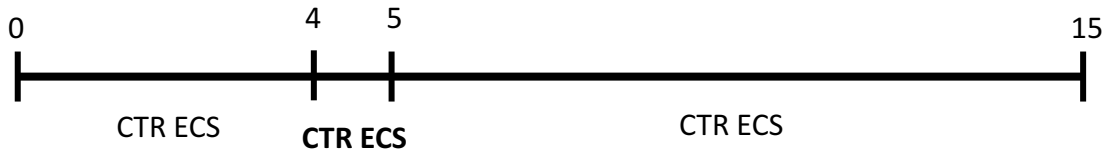
First, it was important to establish whether the observed activity was stable over time, to rule out the possibility of changes in the network activity caused solely by the long recordings. To test whether there is any decay in the observed spontaneous neuronal activity over longer recording periods, we conducted two 15 min recordings on the same sets of cells. During the first recording we continuously applied CTR ECS for the entirety of 15 min. Immediately after the acquisition of the first recording, we initiated the second recording, consisting of the application of 4 min of CTR ECS, then 1 min of 100nM PA and back to CTR ECS for 10 min washout period.

In the experiments we focused mainly on the calcium peak frequency as the parameter that represents the neuronal firing rates. During the control recording, we didn't observe any significant change in the calcium peak frequency, which is essential for the further use of this experimental system. As was expected, 1 min application of 100nM PA caused a significant decrease in calcium peak frequency compared to the first control recording (Figure 13). The calcium peak frequency (mean value from min 5 – 8) after the 1 min application of 100nM PA was 50 ± 5 % of the normalized baseline frequency, which was significantly different compared to the 100 ± 14 % after the application of CTR.

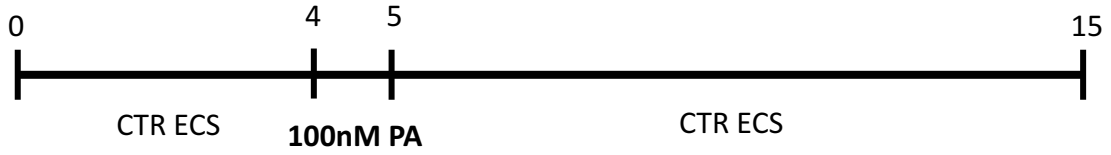
To ensure we could achieve as complete washout of the steroid as possible, we prolonged the washout period to 10 min, making the recordings 15 min in total. Thanks to the longer washout we saw an improvement in the return of the calcium peaks compared to that presented in Figure 12. At the same time, the results from the two 15 min recordings showed that we can observe stable network activity even at longer time scales.

Application timeline (min):

1st recording:



2nd recording:



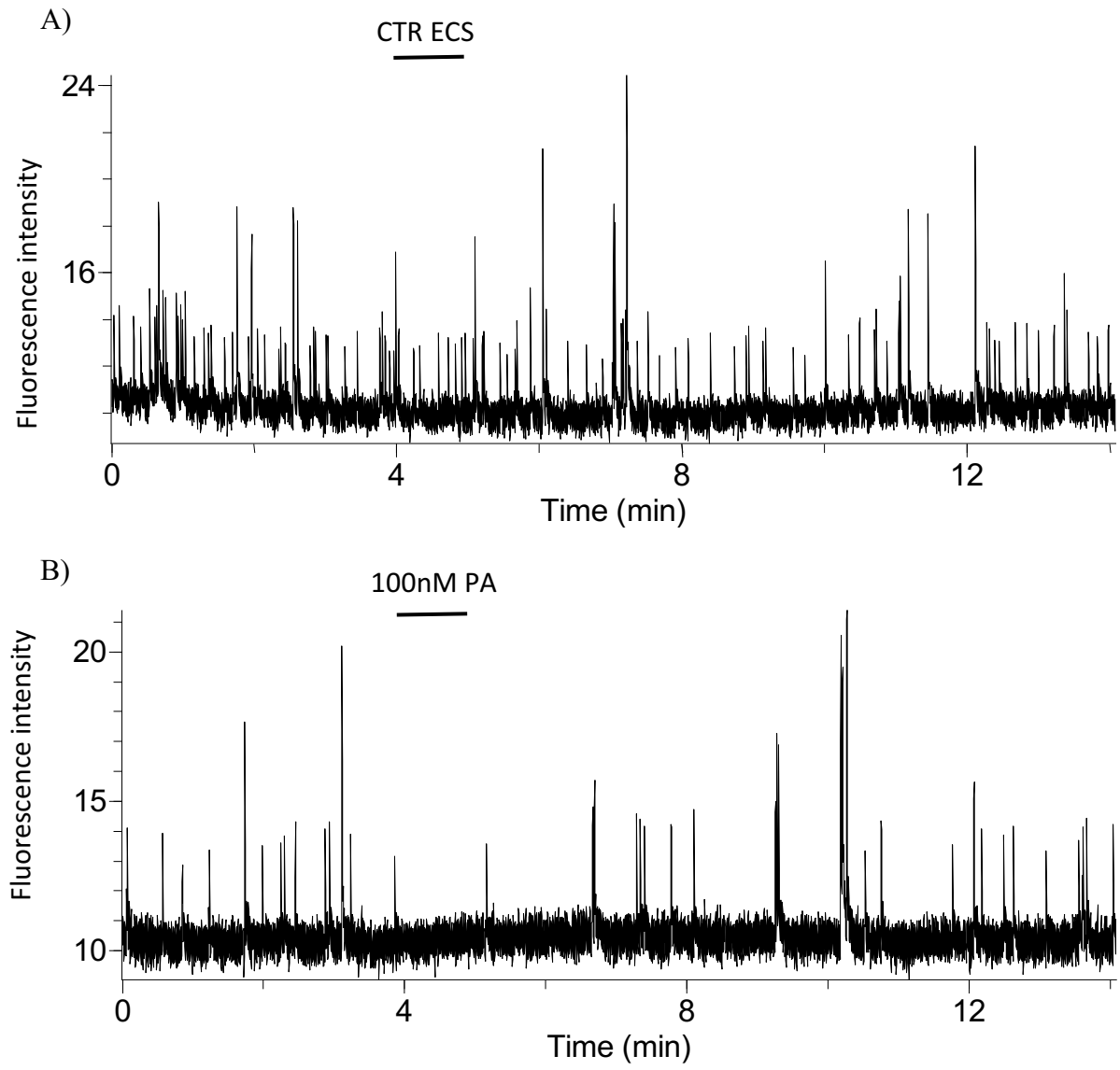


Figure 13: Application of neurosteroid after 15 min CTR recording exhibits stable effects on neuronal activity. A) During the 15 min application of CTR ECS we did not observe any noticeable difference in the calcium peak frequency. **B)** After the 15 min application of CTR ECS, we observed a robust effect of 1 min application of 100nM PA on the network activity. PA caused a major decrease of calcium peak frequency, comparable to the one demonstrated in Figure 12 and which was in contrast to the lack of inhibition during the first recording. Total length of the second recording was also 15 min, with 4 min CTR ECS, 1 min 100nM PA and 10 min of CTR ECS washout period. **C)** Figure continues on the next page.

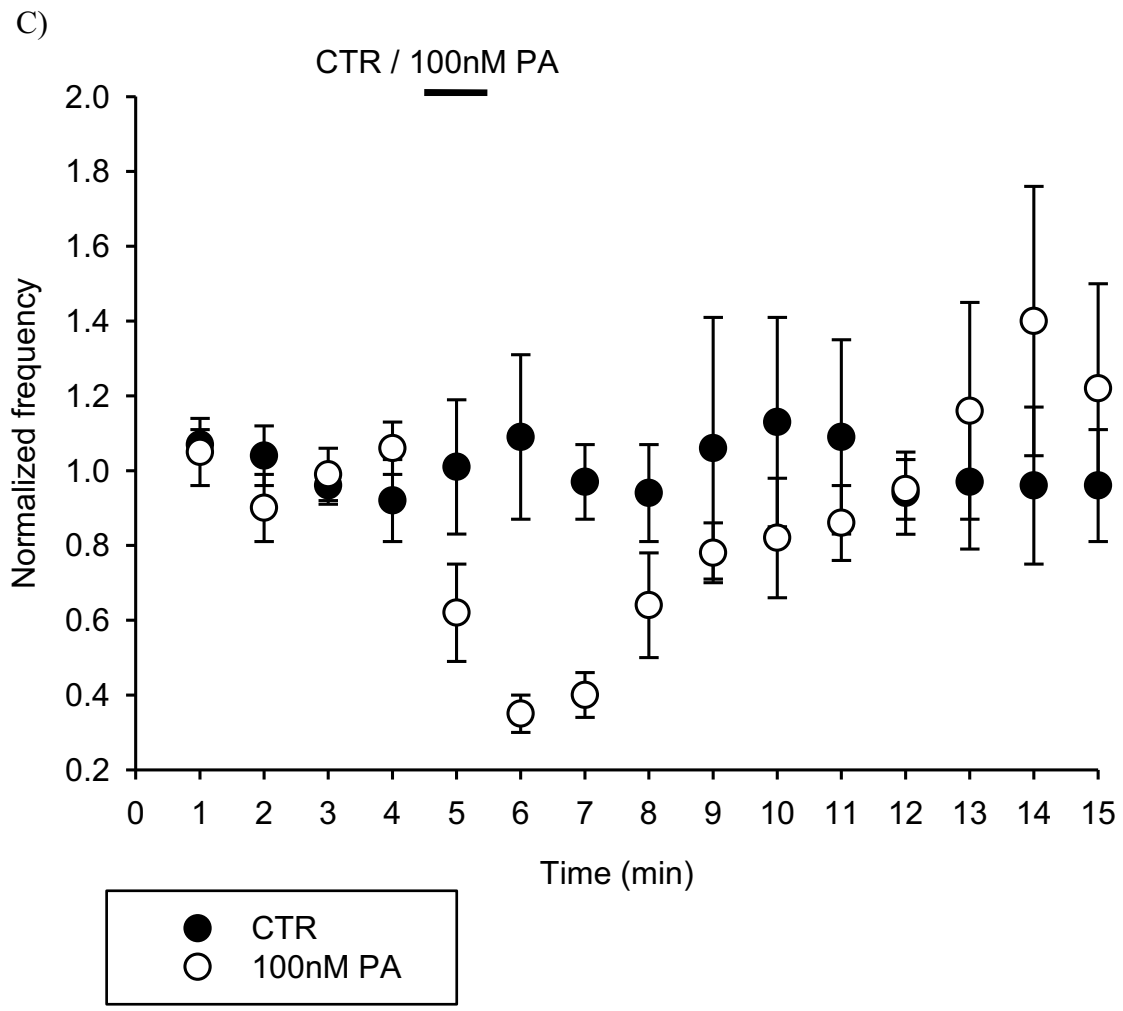


Figure 13: C) The application of 15 min CTR resulted in no significant decrease of the calcium peak frequency, compared to the second 15 min recording where application of 100nM PA caused a robust decrease in the peak frequency. During the washout period, the calcium peaks returned to their baseline levels, although we could still observe some variability between the cells in terms of the peak frequency **D)** Figure continues on the other page.

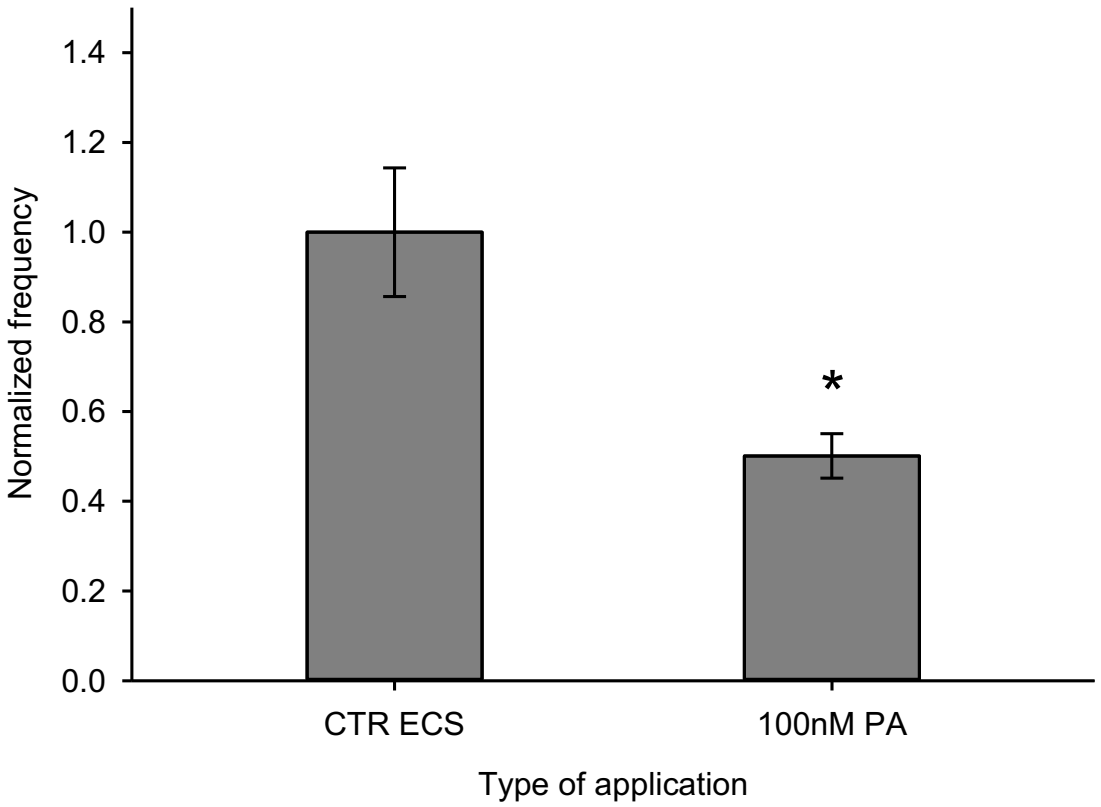


Figure 13: D) There was a significand difference between the frequency of calcium peaks after the application of CTR ECS in the first recording and after 1 min of 100nM PA in the second recording. The mean calcium peak frequency after ECS was 100 ± 14 %, whereas after the application of neurosteroid it was 50 ± 5 % (mean value of frequency was taken from min 5 – 8). Paired t-test showed a significant difference between the two recordings ($P \leq 0.05$; $n = 11$).

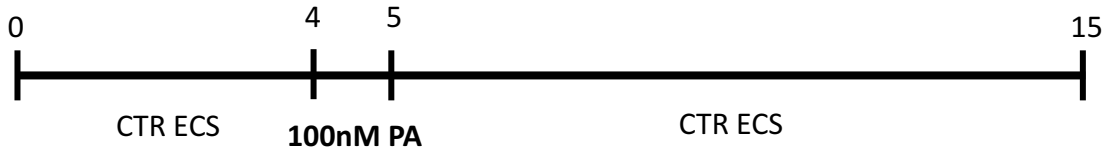
9.4. Two repeated applications of 100nM PA differently affect neuronal activity.

Finally, we tested the effects of repeated application of 100nM PA on neuronal network activity. As described above, we conducted two 15 min recordings with the same sets of cells, this time identical in experimental design. In both recordings we first applied 4 min of CTR ECS, then 1 min of 100nM PA and finally 10 min of CTR ECS to ensure the neurosteroid washout from the culture.

Interestingly, when we compared the level of inhibition of the neuronal activity and the dynamics of the neurosteroid washout between the two recordings, we observed some significant differences. In the first recording, the application of 100nM PA had a seemingly quicker onset of action and during the subsequent minutes of the washout, the neuronal activity returned more slowly, in contrast to the second application of 100nM PA (Figure 14A, B and C). The mean frequency of calcium peaks from minutes 5 – 12 in the first recording was 48 ± 8 % of the baseline value, whereas in the second recording it was 76 ± 9 % (Figure 14D). It appears that the effect of the second neurosteroid application is a bit diminished compared to the first application. Due to the experiments with the application of PA after a 15 min CTR, where we could observe the effect of neurosteroid well, we can eliminate the possibility that the effect of PA in the second recording was effected by the length of the experiment.

Application timeline:

1st recording:



2nd recording:

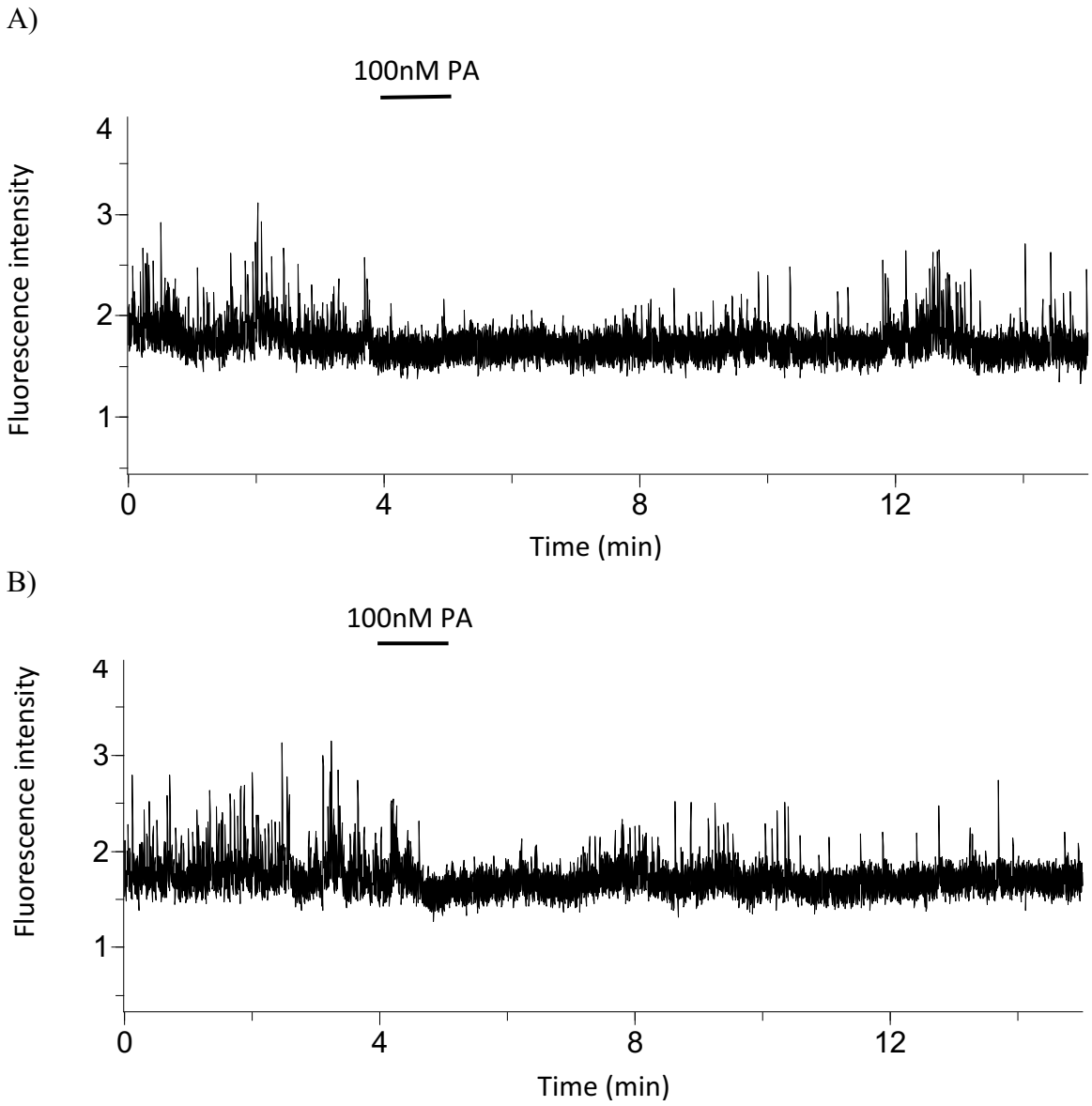


Figure 14: Two repeated applications of 100nM PA to the same sets of cells differ in the inhibition of the calcium peaks. Example traces from one cell of the **A)** first and **B)** second recording. Both of the 100nM PA applications substantially inhibited the frequency of calcium peaks, which gradually returned to its basal level. We observed a difference between the two recordings in terms of the PA-mediated inhibition and the dynamics of the neurosteroid washout. Total length of the recording was 15 min, with 4 min CTR ECS, 1 min of 100nM PA and again CTR ECS for 10 min. **C)** Figure continues on the next page.

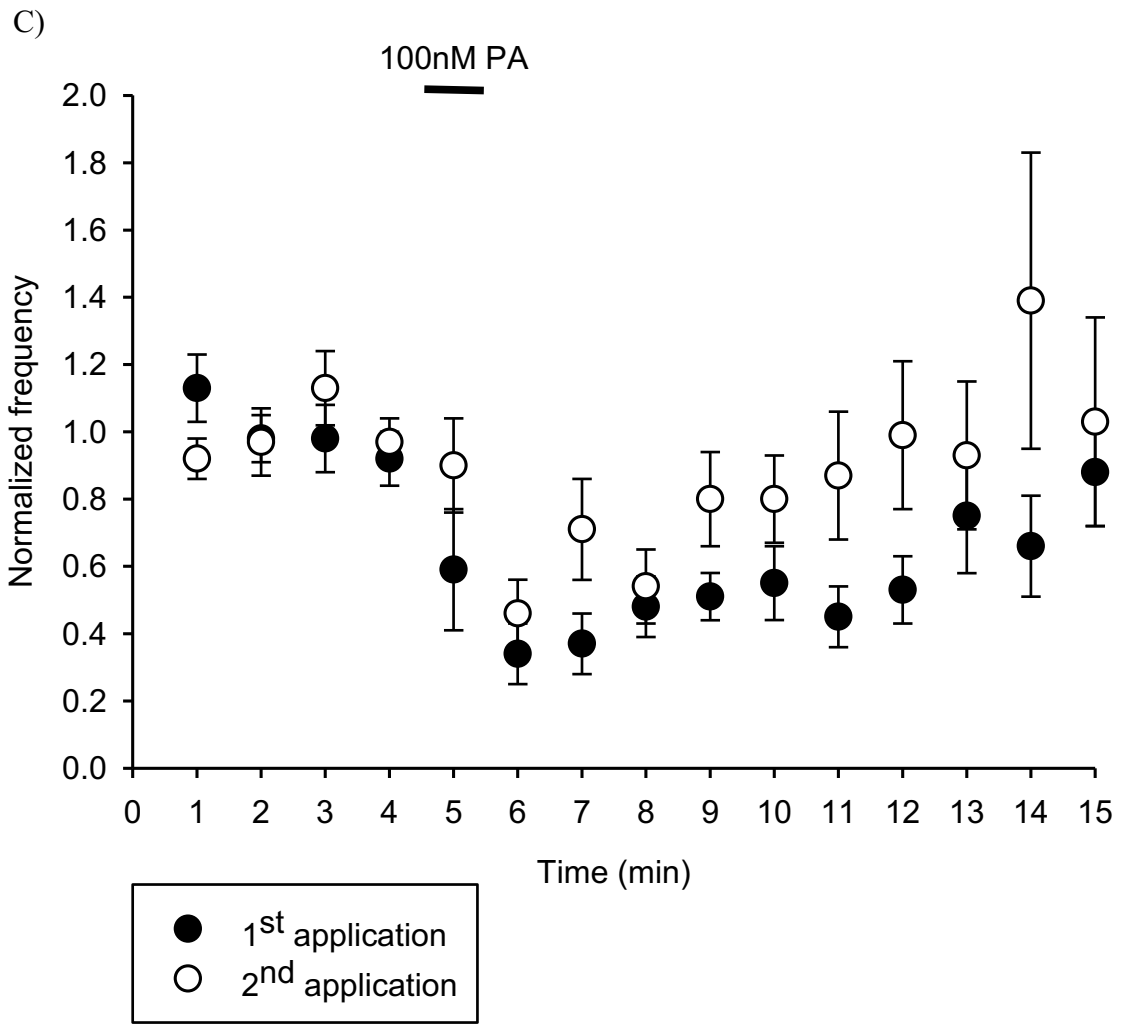


Figure 14: C) Two repeated applications of 100nM PA exhibit different time course of action. The two applications were always conducted with the same set of cells. Once the first recording was finished, we immediately started the second one. In the second 100nM PA application, the neurosteroid ability to inhibit the calcium peak frequency was to some extent diminished compared to the first recording. Moreover, the washout of the neurosteroid and the return of the calcium peak frequency to the basal level seemed to be faster in the second recording. Values of calcium peak frequency were normalized to the mean value of the first 4 min of the CTR ECS application. Mean values of n = 11. **D)** Figure continues on the next page.

D)

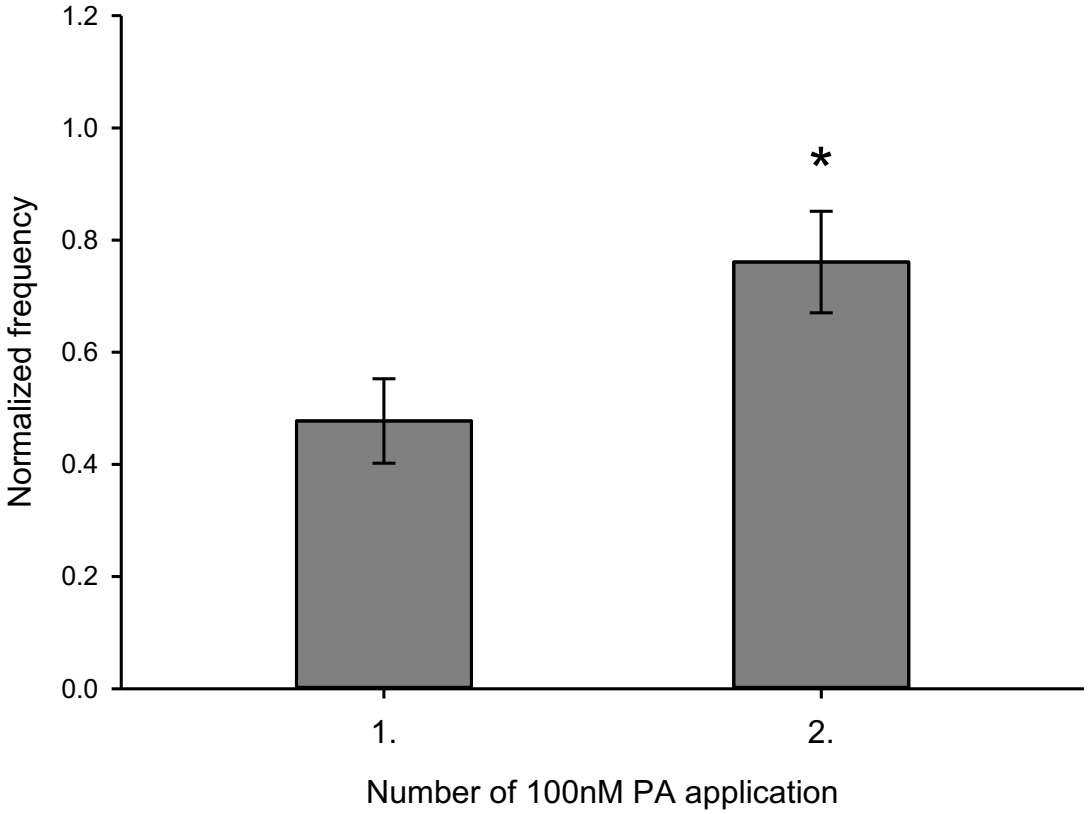


Figure 14: D) The level of inhibition of the network activity during the second application of 100nM PA was significantly differed from the first neurosteroid application. The calcium peak frequency during the first PA application was 48 ± 8 % of the baseline peak frequency, whereas during the second recording it was 76 ± 9 % of the baseline frequency (the peak frequency was calculated as a mean of the frequency from min 5 – 12). Paired t-test showed a significant difference between the two recordings ($P \leq 0.05$; $n = 11$).

10. Discussion

In this work we established a newly assembled application system and tested the method of calcium imaging using the genetically encoded calcium indicator jGCaMP7f. Then we examined the effects of the $GABA_AR$-potentiating neurosteroid pregnanolone on the network activity of primary hippocampal mass cultures.

First, we validated the application system and the calcium imaging method as a useful tool to measure the activity of neuronal networks in vitro. To accomplish that, we used a 2 min application of 0.5µM TTX to the neuronal cultures. TTX is a toxin that is commonly used in electrophysiology, pharmacology and various live-cell imaging experiments. It blocks the sodium voltage-dependent channels, which prevents the production of action potentials (Narahashi et al., 1964). Application of 0.5µM TTX resulted in a complete disappearance of the observed spontaneous calcium peaks, which then gradually returned to their basal frequency and amplitude during the washout period. This experiment proved that the observed calcium peaks reflect neuronal electrical activity.

Second, we compared the inhibition of network activity by 2 min application of 100nM PA using two different types of recording methods, a patch-clamp electrophysiology in the current-clamp configuration and a GCaMP fluorescence imaging. As seen from the example traces in Figure 12, PA caused a comparable level on network activity inhibition. However, the return of the activity to the basal values was slower in the calcium imaging experiments compared to the patch-clamp recordings. This could be caused by technical differences in the application system or in the rate of exchange of solutions from the petri dish with the grown cells.

It has been previously demonstrated, that cultured rat hippocampal neurons express different subtypes of $GABA_AR$, including the δ-containing receptors (Brooks-Kayal et al., 1998; Killisch et al., 1991; Mangan et al., 2005), and the GABAergic inhibitory interneurons account for an important subpopulation in the hippocampus. Although the GABAergic interneurons represent only around 10 % of the total number of hippocampal neurons, they are essential for the regulation of circuit function and neuronal excitability (Benson et al., 1994; Pelkey et al., 2017). The observed inhibition of neuronal activity by PA therefore aligns with the known effects of PA as a positive modulator of $GABA_AR$.

Next, we examined two consecutive recordings, former being an application of CTR ECS and the latter containing a 1 min application of 100nM PA, to test if there is any rundown or other important changes in the visible spontaneous activity of the hippocampal culture during our recordings. The results showed that compared to the CTR application, 1 min of 100nM PA caused a major inhibition of neuronal network activity, which then returned to its basal values. During the control 15 min recording we did not observe any considerable changes in the calcium peak frequency, apart from small noticeable variety between the one-minute intervals we used in the result analysis. The calcium peak frequency after the application of CTR was 100 ± 14 % of the baseline values, the frequency of the peaks after the application of steroid was 50 ± 5 % of the baseline. With this experiment we confirmed that this type of experimental setup is suitable for longer applications and recordings to observe the activity of neuronal cultures on longer timescales.

Lastly, we compared two repeated applications of 100nM PA in two consecutive 15 min recordings. The application of 100nM PA caused a major inhibition of neuronal activity in both recordings, but we could also observe a significant change between the two applications. After the first PA application, the calcium peak frequency was 48 ± 8 % of the baseline peak frequency, whereas after the second it was 76 ± 9 %. The inhibition had a later onset and it returned to the basal levels during the washout period faster compared to the first recording.

It will be necessary to conduct more control experiments as the next steps following the completion of this work. Since we have not examined applications of the neurosteroid together with GABA, the future experiments could test the effects of repeated applications of GABA alone and of GABA together with the neurosteroid, with different concentrations of both of the applied compounds. Another useful point would be to observe the effects of other $GABA_AR$ modulators with a more in depth characterized mechanism of action, such as benzodiazepines and barbiturates, in repeated or prolonged applications. With these experiments we could better understand whether and how does the neuronal network adapt to the changes in activity levels from various causes.

The observed changes in the neuronal activity after repeated PA applications were significant, however, since the washout period was long we could have missed some other changes in the network activity in the earlier minutes of the washout. The experiments could

be designed in a different manner to better examine these earlier min after the applications of steroid.

A possible mechanism behind the observed effects of the repeated PA applications might be the $GABA_AR$ internalization. The phenomenon of neurosteroid-mediated regulation of expression and trafficking of $GABA_AR$ has been described in several studies using mice models (Maguire et al., 2009; Maguire & Mody, 2008). The researchers described a significant decrease in both tonic and phasic neuronal inhibition in pregnant mice, which was mediated by the downregulation of $GABA_AR$ δ and γ_2 subunits (Maguire & Mody, 2008). Moreover, mice with decreased levels of the δ subunit exhibited depression-like behaviors (Maguire & Mody, 2008). Other physiological situations where the $GABA_AR$ are internalized, and the level of network inhibition is therefore influenced, are for instance the ovarian cycle and stress (Maguire & Mody, 2007). Due to the dramatically increased levels of pregnane neurosteroids during pregnancy, the process of $GABA_AR$ downregulation serves as a protective mechanism to avoid sedation (Mody, 2019).

To develop highly specialized and effective neurosteroid therapeutics, it is critical to learn more about the prolonged or heightened presence of the $GABA_AR$-modulating neurosteroids in the brain. We need to examine how these compounds effect the neuronal circuitry and how this then translates into the clinical context. The results presented in our work can contribute to this understanding and they are worth further exploration.

Conclusion

In this work we established an application system and a calcium imaging method using the genetically encoded calcium indicator GCaMP to test the effects of neurosteroids on neuronal activity in vitro. The experimental system was validated by using the application 0.5μM TTX, which completely abolished the spontaneously occurring calcium peaks. Then we compared the calcium imaging technique with electrophysiology patch-clamp experiments to verify that the two methods were comparable in terms of imaging the activity of neuronal cultures. We examined the effects of GABAAR-modulating neurosteroid pregnanolone on the network activity of primary rat hippocampal cultures. The 100nM concentration of PA caused a significant decrease in calcium peak frequency, which corresponded to the known effects of PA on GABAAR. Our results demonstrated that even in longer recordings the level of neuronal activity stays consistent, and it is therefore possible to use the GCaMP calcium imaging to study the neurosteroid actions after repeated or prolonged applications. We observed that repeated applications of PA differently modulate the cultural activity which is an effect worth further examination. Overall, we proved that calcium imaging with genetically encoded sensors is a useful tool for the examination of the properties of potential pharmaceutical agents, such as neurosteroids, and can be used as an additional method together with already commonly practiced electrophysiology and behavioral experiments.

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